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Philosophiae Doctor Dissertation

OLEORESINS FROM RED HOT PEPPER

- EXTRACTION AND APPLICATION

by

JANA SIMONOVSKA

M. Ss. in Food Technology and Biotechnology

Mentor: Full Prof. Dr. Željko Knez

Maribor, XX 2016

ANNEX 4

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FAKULTY OF CHEMISTRY AND CHEMICAL ENGINEERING

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- EXTRACTION AND APPLICATION

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Univerza v Mariboru

Fakulteta za kemijo in kemijsko tehnologijo
Univerza v Mariboru

Fakulteta za kemijo in kemijsko tehnologijo, Smetanova ulica 17,
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Summary

Pepper (*Capsicum annuum* L.) as widely distributed vegetable crop in the world is an excellent source of nutritive and biologically active compounds. The characteristic compounds, capsaicinoids and carotenoids, highlight the importance of the red hot pepper varieties and their oleoresin extracts in the food and pharmaceutical industry.

In the Ph.D. thesis was studied the possibility for a separate and integral utilization of the red hot pepper for obtaining the oleoresins from pericarp, placenta, seeds and stalk. Pre-treatment of the raw material (drying, separation of anatomical structures i.e. pericarp, placenta and seeds, and determination of theirs physico-chemical characteristics and determination of the he characteristic bioactive compounds: capsaicinoids, carotenoids and volatiles was studied, also.

The second part of the Ph.D. thesis was focused of the determination of the optimal conditions for isolation of the bioactive capsaicinoids and coloured compounds, through comparative following of the thermodynamical parameters by application of organic solvents and supercritical fluids. Influence of the working parameters: temperature, time, pressure, solid to liquid phase ratio, density, type of solvents, and particle size of raw material on the yield of extract and content of capsaicinoids, colour compounds and volatiles was studied. Modelling of the experimental phase data by application of mathematical methods was performed.

Re-utilization of seed and stalk from red hot pepper in form of extracts for development of new formulations as edible films, biopesticides and nanoemulsions was studied, also.

Key words:

red hot pepper, pericarp, placenta, seed, stalk, extraction, sub- and supercritical fluids, bioactive compounds, volatiles, re-utilization, edible films, biopesticides, nanoemulsions

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SYMBOLS AND ABBREVIATIONS

A	Absorbance
BI	Browning index
CAP	Capsaicin
DHC	Dihydrocapsaicin
DSC	Differential scanning calorimetry
FTIR	Fourier transform infrared ray
GC	Gas chromatography
HS	Head space
MS	Mass spectrometry
$SCCO_2$	Supercritical carbon dioxide
SCFE	Supercritical fluid extraction
TGA/DTA	thermogravimetric analysis
UV	Ultraviolet
WI	Whiteness index
WVP	Water vapor permeability

1. INTRODUCTION

Pepper (*Capsicum annuum* L., *Solanaceae*) is recognized as a widely distributed vegetable crop in the world. The sweet and hot pepper varieties included in the nutrition and food processing industry are consumed either fresh or processed, in various specialties or as spices and extracts. Pepper is an excellent source of proteins, fats and oils, minerals, vitamins, ascorbic acid, phenolic compounds, aromatic substances and other biologically active compounds (Campos et al., 2013). The characteristic compounds, capsaicinoids and carotenoids, emphasize the importance of the red hot pepper varieties and their oleoresin extracts in the food and pharmaceutical industry (Perva-Uzunalić et al., 2004; Guzman et al., 2011).

Pungency, a commercially important pepper characteristic is defined by the capsaicinoids. Capsaicin is predominant in quantity followed by dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin and homocapsaicin (Davis *et al.*, 2007). The interest for the determination and confirmation of the capsaicin activities increased in last twenty years. The activity of the capsaicin in pain relieving (Knotkova et al., 2008), chemoprevention (Surh and Kundu, 2011), body weight regulation through the fat thermogenesis (Reinbach et al., 2009), cardiovascular and gastrointestinal system maintaining (Wang et al., 2011), glucose blood level reducing (Chaiyasit et al., 2009), as well as in haematuria treatment (Uzoh et al., 2008) has been studied. The application of the capsaicinoids as natural product-based food additives, antimicrobial agents (Singh and Chittenden, 2008), and self-defense product constituents (Mendelson et al., 2010) is of high importance. The positive effects of capsaicin on carotenoid deposition in egg yolk, stimulation of the chicken egg production and improving of the broiler body weight (Dougnon and Youssao, 2014) has been confirmed, also.

Carotenoids are responsible for the yellow, orange and red colours of hot pepper fruits. Yellow-orange colours of pepper fruits are mainly due to the accumulation of α- and β-carotene, zeaxanthin, lutein and β-cryptoxanthin. Capsanthin, capsorubin and capsanthin-*5,6*-epoxide are responsible for the deep red colours (Del Rocío Gómez-García and Ochoa-Alejo, 2013). Carotenoids possess a range of important biological activities. They are potent antioxidants acting as scavengers of singlet molecular oxygen, peroxyl radicals and reactive nitrogen species (Hernández-Ortega et al., 2012). The consumption of carotenoid-rich foods reduces the incidence of several disorders such as cancers, cardiovascular diseases, age-related macular degeneration, cataracts, diseases related to compromised immune function, and other degenerative diseases (Perera and Yen, 2007).

Oleoresin of pepper as a common red pepper product is an organic oily resin, slightly viscous, homogenous red liquid extracted from the dried ripe fruits of sweet or hot varieties of *Capsicum annuum* L. Basically, *Capsicum* oleoresin contains capsaicin and pigments

carotenoids predominantly capsanthin. Furthermore, flavors, taste agents, vitamins and fatty oil are also present in the *Capsicum* oleoresin components profile. There are three types of oleoresin: paprika, red pepper and Capsicum used for colour, colour and pungency, and pungency, respectively (Pruthi, 2003; Guzman et al., 2011, Nadeem et al., 2011).

Generally, the most commonly employed and a preferred method for extraction of compounds present in plant matrices is the conventional solid-liquid extraction using organic solvents. In later studies, these conventional methods were improved, modified or rationalized by varying different operating parameters. The red pepper oleoresins are produced by solvent extraction of dried, ground red pepper fruits, using a solvent-system with the lipophilic/hydrophilic characteristics and subsequent solvent-system removal. The solvents most commonly used for paprika oleoresin extraction are trichloroethylene, ethylacetate, acetone, propan-2- ol, methanol, ethanol and *n*-hexane Thee oleoresin is further treated with polar solvent, methanol, in order to separate the pungent component from the colour component of the oleoresin (Boyadzhiev et al., 1999; Bo et al., 2008; Boonkird et al., 2008).

The extraction process of the raw materials with organic solvents has shown some disadvantages: solvents cannot be completely removed, high extraction temperature can cause extract destruction, solvent extraction is energy intensive, leads to problems of toxic waste disposal, gives a product that requires further purification, etc. Thus, new extraction technologies need to be established for enhancing recovery yields, reducing cost and minimize impact on environment and health. Supercritical fluid extraction (SCFE) methods have the potential to address these needed improvements and increase selectivity and purity (Herrero et al., 2010; Capuzzo et al., 2013). The disadvantages overcoming of the conventional extraction techniques is enabled applying supercritical fluid extraction (SCFE), which in the last two decades has been received as “green-eco” technology and promising technique for extraction of natural products. The application of the SCFE eco-technology in the production of the extracts with high purity and toxicological safety is of particular importance compared with liquid solvents, supercritical fluids have several major advantages: (1) the dissolving power of a supercritical fluid solvent depends on its density, which is highly adjustable by changing the pressure or/and temperature; (2) the supercritical fluid has a higher diffusion coefficient and lower viscosity and surface tension than a liquid solvent, leading to more favorable mass transfer (Reverchon and De Marco, 2006). The most significant use of supercritical carbon dioxide ($SCCO_2$) is supported by its chemical inertness, it is non-toxic, non-hazardous and non-flammable and it is easily removed from the products, it has a convenient critical temperature and enables extraction to be carried out at comparatively low temperature decreasing the risk of damage of thermalabile compounds. Most of the volatile components, which tend to be lost in hydrodistillation, are present in the

supercritical extracts, as well as, the possibility of transferring technology developed in the laboratory/pilot plants in the industrial scale is respectable (Wang and Weller, 2006; Sapkale et al., 2010; Oman *et al.*, 2013).

The content of nutritional and biological active compounds in the fruits of red hot peppers may vary considerably depending on numerous factors, i.e. fruit structural characters, degree of ripeness, environmental conditions, time of vegetation, soil conditions, genetic factors as well as the country of origin (Mansour-Gueddes et al., 2012; Rahman and Inden, 2012; Victoria-Campos et al., 2015). The lack of numerous literature data related to the content of nutritional and biologically active compounds in the fruits of hot varieties of *Capsicum* in the Republic of Macedonia contributed to define the aim of research in the first part of Ph.D thesis, with a focus on determining the quantity of these compounds in the constitutional parts of fruits of red hot pepper, i.e., in the pericarp, seed, placenta and stalk.

Determination of the optimal conditions for isolation of the active compounds from raw materials, through comparative following of the thermodynamical parameters by application of organic solvents and SC fluids is high of importance (Sahena et al., 2009; Da Silva, 2016). Influence of the working parameters: temperature, time, pressure, particle size on the yield of extract and content of capsaicinoids, colour, fatty acids and aromatic compounds from pericarp, placenta, seed and stalk from red hot pepper was researched in this work.

Pericarp is edible part of pepper fruits, while seed, placenta and stalk are waste obtained during pepper processing. Although treated as environmental pollutants, they are recognized as a source of valuable compounds, also. The seed and placenta of red hot pepper are characterized by a valuable content of proteins and micro- and macroelements. In the placenta, in comparison to the pericarp and seed, over 60% of the total quantity of capsaicinoids, capsaicin and dihydrocapsaicin was found (Simonovska et al., 2014). A strong antioxidant and cytostatic activity, as well as anti-inflammatory effects were determined for the stalk extracts due to the high content of phenols, flavonoids and capsaicin (Chen and Kang, 2013). Nowadays, plant origin waste which are accumulated in agro-industrial fields or discarded into rivers causing environmental problems have no significant industrial or commercial uses even they are rich in biological active compounds. Re-utilization of the agriculture and food waste as a bio-resource for the production of novel products is high of impact (Galanakis, 2012; Mirabella et al., 2014). The possibility for re-utilization of extract obtained from seed and stalk of red hot pepper formulated in new products was investigated in this thesis.

2. AIMS OF THE THESIS

In the objectives of the Ph.D. thesis were included:

1. Investigation of the possibility for a separate and integral utilization of the Macedonian red hot peppers for obtaining the oleoresins from pericarp, placenta, seeds and stalk. Pre-treatment of the raw material (drying, separation of anatomical structures i.e. pericarp, placenta and seeds, and determination of theirs physico-chemical characteristics with the attention of the characteristic bioactive compounds: capsaicinoids, carotenoids and volatiles.

2. Determination of the optimal conditions for isolation of the bioactive capsaicinoids and coloured compounds, through comparative following of the thermodynamical parameters by application of organic solvents and supercritical fluids. Influence of the working parameters: temperature, time, pressure, solid to liquid phase ratio, density, type of extraction solvent and particle size of raw material on the yield of extract and content of capsaicinoids and colour compounds was studied. Modelling of the experimental phase data by application of mathematical methods.

3. Re-utilization of seed and stalk from red hot pepper in form of extracts for development of new formulations as edible films, biopesticides and nanoemulsions.

3. STATE OF ART

3.1. PEPPER (*Capsaicum* spp., *Solanaceae*)

3.1.1. Origin and botanical characteristics

Capsicum or Cayenne Pepper was known in the tropics of the American land for thousands of years. Archaeological data testifying for the cultivation and use of pepper before 7000 BC by the indigenous people who lived in the territory south of the Mexican border. In Europe, this kind of culture was brought by Christopher Columbus. During the first trip to America in the period 1492-1943, Columbus noted that the indigenous people of the "New World" used as food red coloured fruits called "aji" or "axi". Newly discovered culture was named as "red pepper aftert transfering in Spain. De Cuneo who accompanied Columbus on his second journey in the "New World " for peppers will write that "there is bush as a rose, with fruit long as the fruits of cinnamon, is hot as pepper, and Caribbeans and Indians eat it as we eat the apple". At the end of the 16th and 17th centuries, in the Mediterranean and central European regions, the cultivation of the pepper was intensified, and for a short time was expanded in the Asian and African continent, in many tropical and subtropical regions worldwide (Basu and Krishna De, 2003; Nunn and Qian, 2010). The true origin of the word *Capsicum* is a mystery. Word *Capsicum* (KAP-sih-kum) is derived from the Greek word "kapto" meaning " to bite" that would be appropriate taking into account the hot taste of the fruit of the plant, and from the Latin word "capsa" concerning the shape of the pepper fruit (Clement, 2010).

Pepper is an annual bush plant that is cultivate in large scales due to the fruit used in food as a vegetable. There are many pepper varieties and cultivars. The pepper fruit is elongated berry, hollow inside, with shiny and smooth pericarp, spiky on top and the base broadened and attached to green–brown stalk. In the lower section on the several placentas are fastened flat, round, yellow seeds (Fig. 3.1). Concerning the degree of ripeness, the fruit of the pepper is with green, red or orange-red colour, low odor. The pungency degree characterized the pepper with sweet or bitter taste. The flavors of the peppers described by sensor analysis were declared as fruity, earthy, smoked, fresh, sweet, and even floral taste. During the pasting or grinding, hot pepper irritates the mucous membranes of the nose, mouth and eyes, causing sneezing, coughing and tears (Seidemann, 2005).

The genus Capsicum is a member of the Solanaceae family, which includes tomatoes, potatoes and tobacco. Capsicum genus is comprised of more than 30 species, out of which five are domesticated and cultivated: Capsicum annuum, Capsicum baccatum, Capsicum chinense, Capsicum frutescens and Capsicum pubescens (Duke et al., 2003). More

than 200 names are used for the peppers, commonly, "chilli pepper", "paprika", "bell pepper", "halapentos", "chitlepin" and "Christmas pappers"

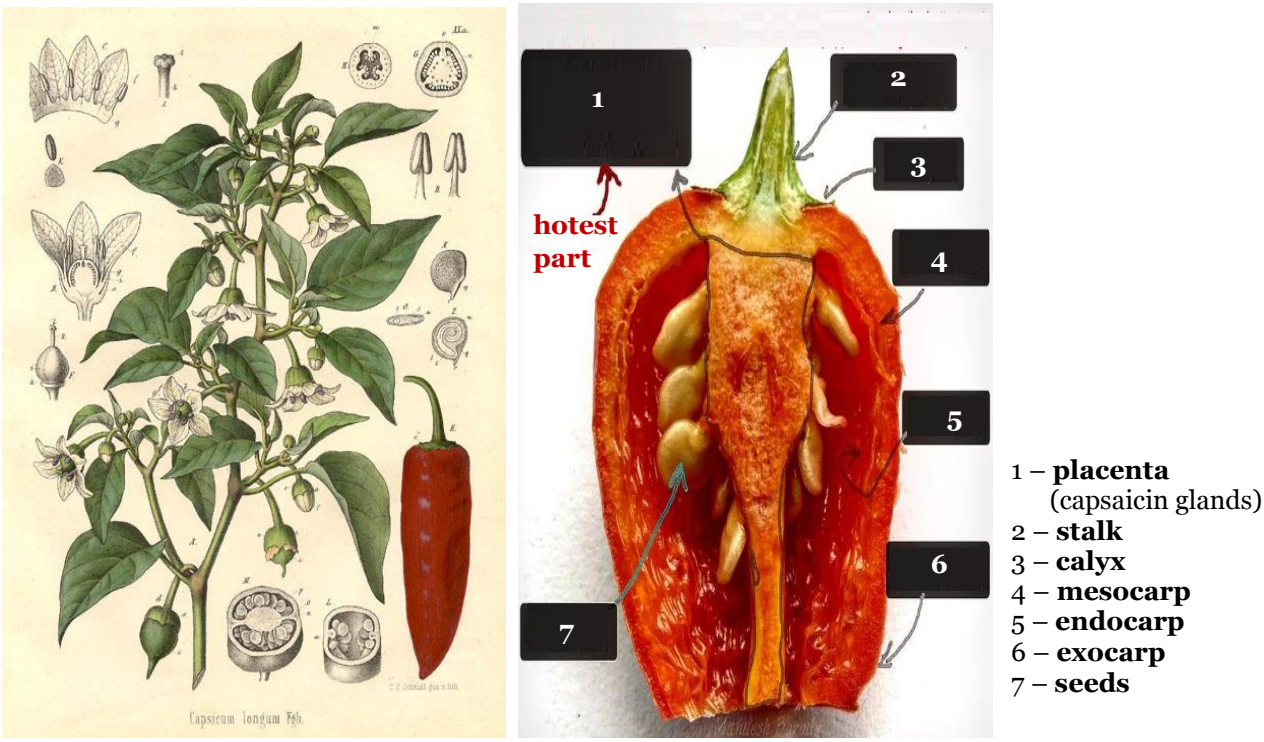


Fig. 3.1. Pepper anatomy and longitudinal section (web 1, 2).

The varieties of the same pepper species can be used in different ways, for example, "bell pepper" which belongs in *Capsicum annuum* is used as food when is green, while in industry when is mature with red, yellow and orange colour. In the *Capsicum annuum* are classified and other varieties as "Anaheim chiles" often used for preparation of homemade specialties, dried "Ancho chile" for chili powder or medium hot "Jalapeño" for product known as "Chipotle" (Seidemann, 2005).

3.1.2. Chemical composition of pepper

The high bioactive compounds content increased the interest in *Capsicum* fruits. The pepper fruits have a characteristic chemical composition. They are consisted of pigments, components responsible for pungency, resins, proteins, pentosans, mineral materials and minor quantities of volatile oils. In the pepper seed high amounts of triglyceride oils was determined. *Capsicum* is one of the best sources of vitamin C containing four times more vitamin C than orange. Teodoro et al. (2013) in the *Capsicum chinense* determined quantities of vitamin C in the range of 54.1 to 129.8 mg/100 g. Phenolics (20.54 to 20.75 mg/100 g sample), carotenoids (1.00 to 1.26 mg/100 g sample) and ascorbic acid contents (187.24 to

281.73 mg/100 g sample) varied between genotypes of *Capsicum chinense* Jacq. var. Habanero (Campos et al., 2013). The conditions for growing and development, as well as the processing after harvesting, caused variations in quantities of pepper compounds. Based on the collected and processed results of over nine hundred laboratories in the United States, Zacharian and Gobinath (2008) reported the following composition of hot *Capsicum* species: 14-16 % protein, 14.10 to 15.50 % fat, 7.20 to 8.00 % ash, 0.10 % calcium, about 0.90 % iron, 0.01 % sodium. In 100 g dry mass, thiamine (0.52-0.59 mg), riboflavin (0.93-1.66 mg), niacin (13.60-14.20 mg), ascorbic acid (29.40 to 63.70 mg) and vitamin A (3.53-6.17 IU) are determined.

According to the content of capsaicinoids, Govindarajan et al. (1986) suggests the following classification of peppers: sweet varieties with 0.1–0.2%, 0.2–0.4% medium hot, hot 0.4–0.6% and very hot varieties to 1.4% capsaicinoids. Contents of capsaicin from 0.003 to 0.01% is characteristic of sweet pepper varieties, while medium hot and very hot are characterized with capsaicin content from 0.05 to 0.3% and 0.3 to 1%, respectively (Perucka and Materska 2003). Sanatombi and Sharma (2008) the fruits of *Capsicum* varieties cultivated in India determined amount of capsaicin from 0.14 to 0.17% in the *Capsicum annuum*, 0.65-0.88% in *Capsicum frutescens* and 0.79–2.06% *Capsicum chinense*. In "Habanero" hot peppers cultivated in Mexico, capsaicin content ranges from 41.8 – 65.9 mg/g dry product (Pino et al., 2007).

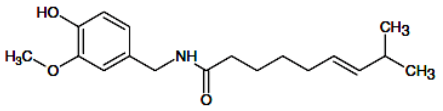
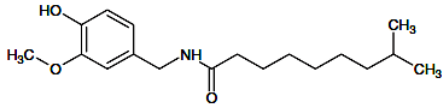
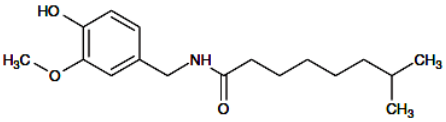
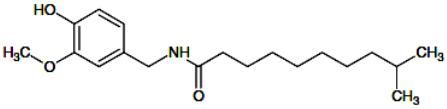
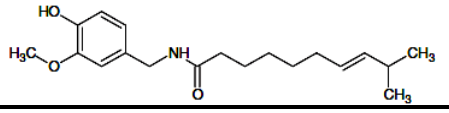
3.1.3. Capsaicinoids: characteristics and biological function

Capsaicin is the active ingredient of hot pepper varieties. Chemically it is an alkaloid that is complex secondary metabolite which is formed in plants as a product of metabolism of the amino acids phenylalanine and tyrosine, actually, the amino acids appear as a precursor in the synthesis of alkaloids. Capsaicin and several related capsaicin compounds are termed by common name capsaicinoids. There are principally five naturally occurring capsaicinoids (Arora et al., 2011): capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin, presented in Table 3.1.

The capsaicin is crystalline-to-waxy solid at room temperature, odorless and white, insoluble in water and easily soluble in fats, oils and alcohols. It is derivate of vanillyl amide consisted of three functional groups: vanillyl aromatic group, acrylamide and aliphatic group (Fig. 3.2). According to IUPAC, capsaicin is 8-methyl-*N*-vanillin-*trans*-6-noneamid with molecular formula $C_{18}H_{27}NO_3$, structural formula $(CH_3)_2CHCH{=}CH(CH_2)_4CONHCH_2C_6H_3$-4-(OH)-3-($OCH_3$), 305,462 g/mol molecular weight, and melting temperature and boiling point of 62-65 ^oC and 210-220 ^oC, respectively (De Lourdes Reyes-Escogido et al., 2011). When

consumed, capsaicin binds with pain receptors in the mouth and throat, which are normally responsible for sensing heat.

Table 3.1. Capsaicinoids (Zachariah and Gobinath, 2008).

Capsaicinoid name	**Relative amount** (%)	**Molecular formula Molecular weight**	**Chemical Structure**	**Scoville heat units**
capsaicin	69	$C_{18}H_{27}NO_3$ 305		16 000 000
dihydrocapsaicin	22	$C_{18}H_{29}NO_3$ 307		15 000 000
nordihydrocapsain	7	$C_{17}H_{27}NO_3$ 293		9 100 000
homodihydrocapsaicin	1	$C_{19}H_{31}NO_3$ 321		8 600 000
homocapsaicn	1	$C_{19}H_{29}NO_3$ 319		8 600 000

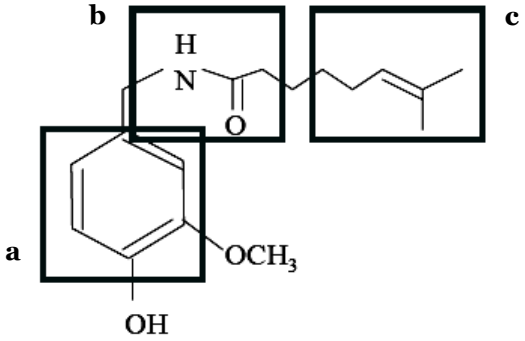


Fig 3.2. Capsaicin: (a) vanillyl aromatic group, (b) acrylamide group and (c) aliphatic groups.

In 1816, capsaicin was first isolated in partially purified crystalline form by Bucholz and in pure crystalline form in 1876 by Thresh, who named it capsaicin. Buchheim was the first who found that capsaicin caused a burning sensation and increased secretion of gastric juice when contacting mucous membranes. The capsaicin structure was partially solved by Nelson in 1919, while in 1930 the compound was originally synthesized by Späth and Darling. Similar substances were isolated from chili peppers by Japanese chemists, who referred to them as capsaicinoids (Cordell and Araujo, 1993). The “heat” degree was measured in so-called "Scoville" units (Scoville Heat Unit, SHU) determined by a *Scoville* method, which was a subjective measure based on organoleptic assessment. Actually, SHU are the number of

times a chili extract must be diluted in water for it to lose its heat. Scoville method was first introduced by pharmacologist Wilburg L. Scoville in 1912 (Scoville, 1912). According to determined Scoville heat values, the following classification of peppers was proposed (Weiss, 2002): sweet non-pungent varieties (0–5 000 SHU), moderately pungent (5 000–20 000 SHU), pungent (20 000–70 000 SHU) and very pungent (70 000–300 000 SHU). Bell peppers rank lowest at 0 SHU, jalapeños and Italian "pepperoncini" score 3 000 to 6 000 SHU, "Thai chiles" reached 100 000 SHU, and habaneros generate 300 000 SHU. Table 3.2. presents the Scoville rating scale of genus *Capsicum* species (web 3).

Table 3.2. *Scoville* rating scale (web 3).

***Scoville* units**	***Capsicum species* or *Capsicum product***
15 000 000 - 16 000 000	pure capsaicin
8 600 000 - 9 100 000	capsaicinoids (nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin)
5 000 000 - 5 300 000	FN 303 pepper spray
855 000 - 1 050 000	Naga Jolokia
350 000 - 580 000	Red Savina Habanero
100 000 - 350 000	Pepper varietis: Habanero chili, Scotch Bonnet, Datil, Rocoto, Jamaican hot pepper, African Birdseye, Madame Jeanette
50 000 - 100 000	Thai pepper, Malagueta pepper, Chiltepin pepper, Pequin pepper
30 000 - 50 000	Cayenne pepper, Ají pepper, Tabasco пипер, Chipotle пипер
10 00 - 23 000	Serrano pepper, Chipotle pepper
2 500 - 8 000	Jalapeño, Guajillo, Mexican varieties of Anaheim pepper, Paprika,
500 - 2 500	Anaheim pepper, Poblano, Rocotillo,
100 - 500	Pimento, Pepperoncini
0	No heat, Bell pepper

Capsaicin biosynthesis in plants is defined by two pathways: (1) the phenylpropanoid pathway derived from phenylalanine leading to vanillylamine, and (2) the branched chain fatty acid pathway derived from valine leading to 8-methyl-6-nonenoyl-CoA. The condensation reaction of vanillylamine with 8-methyl-6-nonenoyl-CoA is catalyzed by a coenzyme A-dependent acyltransferase, namely Pungent gene 1 (*Pun*1) as capsaicin synthase (CS). The capsaicinoid biosynthesis takes place in the glands that are located in the placenta connective tissue that connects the placenta to the fruit pericarp. Capsaicinoids are very quickly accumulated and separated by the membrane, and when they will exceed threshold levels of accumulation then released from the inside out and outside in the form of soluble oils. The capsaicinoids are concentrated only in epidermal cells of placenta of hot pepper (Vázquez-Flota et al., 2007; Ogawa et al., 2015).

Biological function of capsaicin. Capsaicin acts by binding to transient receptor potential vanilloid 1 (TRPV1) who is member of the transient receptor potential

(TRP) family which is a heterogeneous group of non-selective cation channels. TRPV1 is located primarily in the small fibers of nociceptive neurons. Also, TRPV1 is distributed in tissues of the brain, bladder, kidneys, intestines, keratinocytes of epidermis, glial cells, liver, and polymorphonuclear granulocytes, mast cells, and macrophages. TRPV1 contains 838 amino acids and has a molecular weight of 95 kDa in humans. It is a ligand-gated non-selective cation channel, which is activated by multiple stimuli, like heat (>43 °C), voltage, pH lower that 5.9, endogenous lipid molecules, and exogenous agonists, such as capsaicin. TRPV1 regulates intracellular calcium levels by coupling with a nonspecific cation channel permeable to sodium and calcium ions. It is located in the plasma membrane and the endoplasmic reticulum. A heat sensitive subunit of TRPV1 is responsible for burning sensation caused by capsaicin. When capsaicin binds to TRPV1, there is an increase in intracellular calcium which triggers the release of neuropeptides such as substance P and the calcium gene-related peptide (CGRP). Binding between capsaicin and sensory neurons produces pain, inflammation and a localized heat sensation, Fig. 3.3. When applied topically, it desensitizes the sensory neurons skin due to depletion of substance P thus causing analgesic action (Wang et al., 2011; Sun et al., 2016).

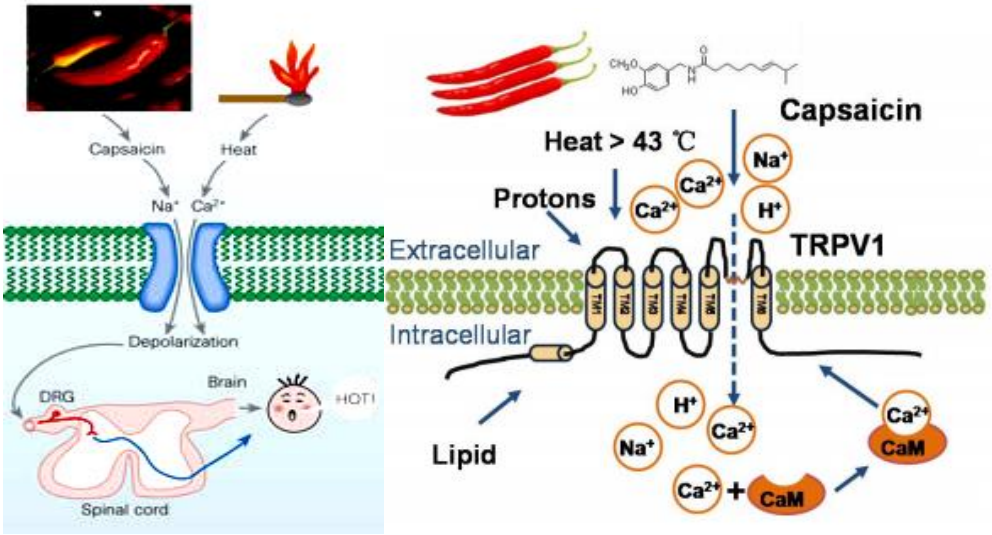


Fig. 3.3. Structural and physiological function of TRPV1 and capsaicin in biological systems (Caterina et al., 1997; Sun et al., 2016).

Pharmacological actions of capsaicin have been known since long ago, but in the last twenty years, particular attention was paid to determine and confirm its specific activity. The TRPV1 activation by capsaicin is favorable for the controlling of cardiovascular diseases, obesity and diabetes mellitus, as well as in cancer prevention and in pain relief. Activation of TRPV1 by dietary capsaicin plays a critical role in the regulation of lipid and glucose metabolism and vascular function including the promotion of lipolysis, the improvement of vasodilation, the upregulation of insulin levels, inhibition of foam cell

formation, and the suppression of adipogenesis. Accordingly, dietary capsaicin can relieve fatty liver, atherosclerosis, hypertension, diabetes, and obesity. Dietary capsaicin has potential benefits for cardiometabolic diseases in the population, Fig. 3.4 (Sun et al., 2016).

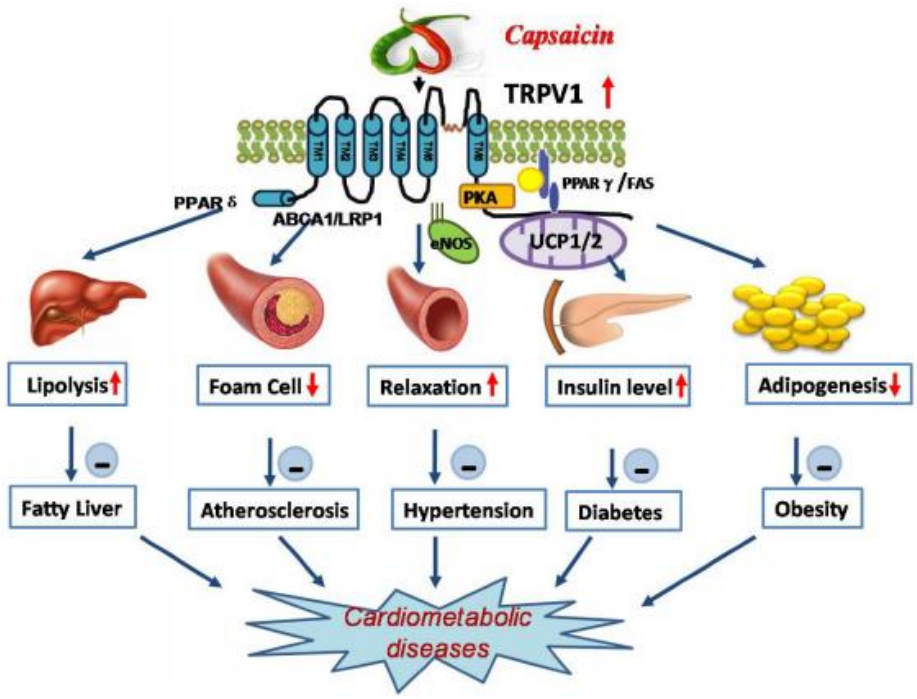


Fig. 3.4. Favorable effects of capsaicin on cardiometabolic disease or related target organ damage (Sun et al., 2016).

Capsaicin used orally or locally reduces inflammation or increased sensitivity to pain caused by chemical agent, increased temperature, or mechanical damage, or reduce the pain of rheumatoid arthritis (Knotkova et al., 2008). The capsaicin acting as a long-acting analgesic to treat post-surgical and osteoarthritis pain was confirmed (Remadevi et al., 2008). Topical capsaicin treatment on patients with diabetic peripheral neuropathy significantly relieved the pain at 12 weeks by stimulation of capsaicin-sensitive afferent nerves (Kiani et al., 2015). In obesity control, TRPV1 activation by capsaicin influences the thermogenesis of the brown adipose tissue and reduces body fat, thus increase in energy expenditure, lipid oxidation and sympathetic nervous system activity, decrease appetite and subsequent protein and fat intake (Ludy et al., 2012). TRPV1 may promote insulin secretion via its calcium influx activity in β-cells, subsequently, dietary capsaicin significantly decreased fasting glucose/insulin and triglyceride levels, as well as the expression of inflammatory adipocytokine genes (Akiba et al., 2004). Activation of TRPV1 by capsaicin exerts an anti-hypertension effect by releasing of calcitonin gene-related peptide (CGRP) from capsaicin-sensitive nerves and nitric oxide from endothelial cells. Transient CGRP increase in the plasma accompanied by a decrease in blood pressure was determined at acute

administration of capsaicin (Deng and Li, 2005). In the cultured cells, capsaicin is able to block the migration of breast cancer cells and to kill prostate cancer cells. Capsaicin was found to inhibit the growth of leukemic cell and isoform of enzyme cytochrome P450 involved in the detoxification of many low-molecular-weight carcinogens (Choi et al., 2010). Capsaicin activating vanilloid receptor-1 increases the release of CGRP from sensory neurons, futher, CGRP increases production of insulin-like growth factor-I (IGF-I) which plays an important role in hair growth. Combined administration of capsaicin and isoflavone promote hair growth by increasing of IGF-I production in hair follicles (Naoiki et al., 2007). Capsaicin as natural preservative expresses significant activity against the yeast *Saccharomyces cerevisiae* (Kurita et al., 2002). In combination with *Lactobacillus casei*, capsicum extracts is effective against wood-discolouring fungi S*phaeropsis sapinea* and *Leptographium procerum (*Singh and Chittenden, 2008*)*. The capsicum oleoresin extracted from *Capsicum frutescens* due to its greater degree of pungency and better penetration into the cellular structure of the woods is effective in preservation of *Pinus* sp. and *Hymenaea* sp. woods from from fungus *Paecilomyces variotti* (Ziglio and Gonçalves, 2014). Padilla et al. (2000) studied the application of the capsaicin as a local anesthetic in dentistry to treat patients with chronic neuropathic disorders. Capsaicin positively affect the absorption and deposition of carotenoids in the egg yolk of chickens nourished with corn fortified with capsaicin. Red hot pepper introduced into the diet of chickens stimulates the formation of the genitalia by development of hormones FHS and LH, thus affects the process of producing eggs in chickens (Őzer et al., 2005).

Other utilization of capsaicin. Capsaicin except as a pharmacological agent, is an active ingredient in “pepper spray” agent. When the spray comes in contact with skin, especially eyes, it causes pain and the person is disabled for a short period of time sufficient police to arrest him. Although spray is not toxic, in large quantities it can cause wheeze and dizziness (Broadstock, 2002). *Capsicum annum* extract were utilized in controlling of the mosquitoes. Larvicidal efficacy during 18h is high of importance at prevention of widespread disease malaria in the world (Madhumathy *et al.*, 2007).

Toxicological aspects of capsaicin. Regardless of all the positive effects of capsaicin, depending on the amount of intake, capsaicin causes histopathological and biochemical changes including erosion of gastric mucosa and lesions of liver and kidneys, after oral ingestion. Although rare, allergic reactions associated with the use of *Capsicum* or its products can occur. It is advisable to stop consumption of hot pepper and seek medical attention if symptoms of allergies, including difficulty breathing, swelling of the lips, tongue, face and urticaria occur. At stomach pain, tachycardia, diarrhea, etc., assistance of doctor or pharmacist is recommended. Capsaicin, is not suitable for treating patients with hematuria (the presence of red blood cells in the urine) due to the negative side effects that can cause

(Uzoh et al., 2008). In the application of topical cream containing capsaicin combined with hot water at shower, capsaicin may cause thermal pain. In the cream containing capsaicin, glyceryl trinitrate in the formulation is required (Walker & McClane, 2002). Carcinogenicity of the capsaicin has been studied and it was found that it did not show carcinogenicity effect in mice at a dose up to 375 mg capsaicinoids/kg body weight/day. Depending on the administration method, the lethal dose in terms of body weight (bw) is 0.56 mg/kg bw intravenously, 190 mg/kg bw orally and 512 mg/kg bw topically (Committee of Experts on Flavouring Substances, 2005). Scientific Committee for Food for capsaicinoids suggested daily intake ranges from 0.5 to 4 mg/kg bw. It also sets the limits for the content of capsaicinoids in products and 5 mg/kg in food and beverages, 10 mg/kg in bitter food and beverages, 20 mg/kg for hot ketchup and 50 mg/kg for tabasko hot Pimenta oils and similar products. The daily intake of capsaicinoids in the US and Europe of industrially prepared food containing capsaicinoids (5 mg/kg product) range from 0.77 to 2.64 mg/kg bw. According to European Commission Decision 1999/217/EC, 1999, and the amendment 2002/113/EC 2002, capsaicin was included in the register of chemicals defined as aromatic substances (European Commision, Health, & Consumer Protection Directorate-General, 2002).

3.1.4. Carotenoids: characteristics and biological function

Carotenoids are lipid-soluble pigments widely distributed in nature that are responsible for the yellow, orange and red colours of plants, algae and fungi. The animals cannot synthesize the carotenoids. The name carotenoid originated from the carrots (*Daucus carota*). The carotenoids as most complex class of natural food colourants that based on a C40 tetraterpenoid skeleton are divided into two groups: hydrocarbons known as *carotenes* and oxygenated compounds generally named as *xanthophylls*. In nature, the carotenoids can be present as free carotenoids or in a more stable form esterified with fatty acids, in the case of the oxygenated compounds. Thermal and photochemical stabilities, as well as the antioxidant capacity are important attributes of the carotenoid esters (Cazzonelli et al., 2011).

In general, carotenoids are consisted of eight isoprenoid units (ip) which compose the C40 carbon skeleton (Fig. 3.6). The main characteristic of carotenoid structure is the long chain consist of single and double bonds that gives the differences in chemical reactivity and light absorbing properties. Further, the activity of carotenoids differs depending upon the size of ring substituents. All carotenoids can be considered as lycopene ($C_{40}H_{56}$) derivatives by reactions involving: (1) hydrogenation, (2) dehydrogenation, (3) cyclization, (4) oxygen insertion, (5) double bond migration, (6) methyl migration, (7) chain elongation and (8)

chain shortening (Delgado-Vargas et al., 2000; Dutta et al., 2005). The important physical and chemical properties of carotenoids are show in Fig. 3.6.

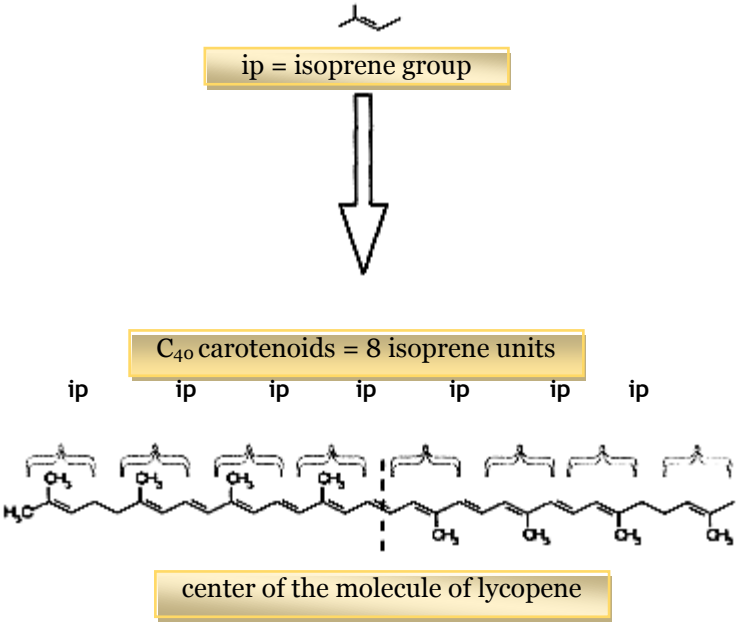


Fig. 3.5. Structure of carotenoids Delgado-Vargas *et al.*, 2000).

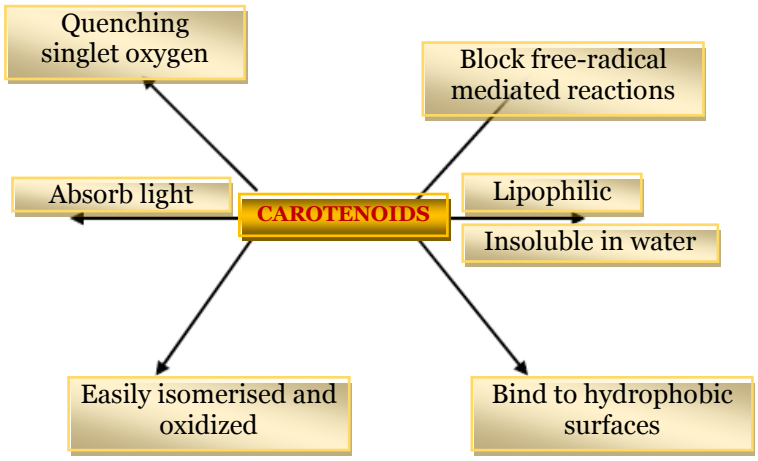


Fig. 3.6. Important physical and chemical properties of carotenoids (Dutta et al., 2005).

The classification of carotenoids depends of the function on the cell level is followed: primary carotenoids like *β*–carotene, violoxantin and neoxanthin required by the plants in photosyntehis process, while, the secondary carotenoids are localized in fruits and flowers (*α*–carotene, *β*–cryptoxanthin, zeaxanthin, antheraxanthin, capsanthin, capsorubin Most carotenoids absorb maximally at three wavelengths, resulting in a three-peak spectrum. As the number of conjugated double bonds increases, the λmax shifts to longer wavelengths.

The most unsaturated acyclic carotenoid*, lycopene*, with 11 conjugated double bonds, is red and absorbs at the longest wavelengths (λmax at 444, 470, 502 nm), while *ζ-Carotene* as light yellow acyclic carotene with seven conjugated double bonds at the shorter wavelengths, λmax at 378, 400, 425 nm (Britton and Khachik, 2009; Saini et al., 2015).

The stability of carotenoids differs in different foods, even when the same processing and storage conditions are used. Alteration or loss of carotenoids during processing and storage of foods occurs through physical removal (e.g.,peeling), geometric isomerization, and enzymatic or non-enzymatic oxidation as a major cause of carotenoid destruction. Isomerization of *trans*-carotenoids to the *cis*-isomers, particularly during heat treatment, alters their biological activity and discolours the food. The enzymatic degradation of carotenoids may be a more serious problem than thermal decomposition in the foods (Pérez-Gálvez *et al.*, 2005). Blanching at temperature under may provoke some losses of carotenoids, but the inactivation of oxidative enzymes that occurs in this type of heat treatment prevents further and greater losses during holding before thermal processing, slow processing, and storage. Generally, freezing and frozen storage at lower temperature (4^{o}C to -20^{o}C) can preserve carotenoids. At traditional sun-drying and drying in electrical dryiers, carotenoids considerable are destructed, while at solar dryers appreciably reduce in carotenoids losses was determined. Food protecting from direct sunlight and treatment with antioxidant and sulfiting agents positively influence on the carotenoid retention. Exclusion of oxygen through vacuum or hot filling, oxygen-impermeable packaging and inert atmosphere, as well as protection of light at low temperature can lower the carotenoid decomposition in food during storage (Boon *et al.*, 2010; Saini et al., 2015, Martins et al., 2016).

Biological activity of carotenoids. The carotenoids are remarkable for their wide distribution, structural diversity, and various functions. They playing an important role in biological systems, starting with light protection in photosynthetic membranes (Cazzonelli, 2011), in the immune response systems (Rao and Rao, 2007, Woodside et al., 2015), in protection against carcinogens (Linnewiel-Hermoni et al., 2015), in cardioprotection (Agarwal *et al.*, 2012), as well as they are characterized with significant antioxidant activity (Han *et al.*, 2012; Martins et al., 2016). Schematic illustration of the possible mechanism and the role of the carotenoids in the prevention of chronic diseases (Veeramachaneni and Wang, 2009) and their biological actions (Rao and Rao, 2007) are summarized in Fig. 3.7 and 3.8 respectively.

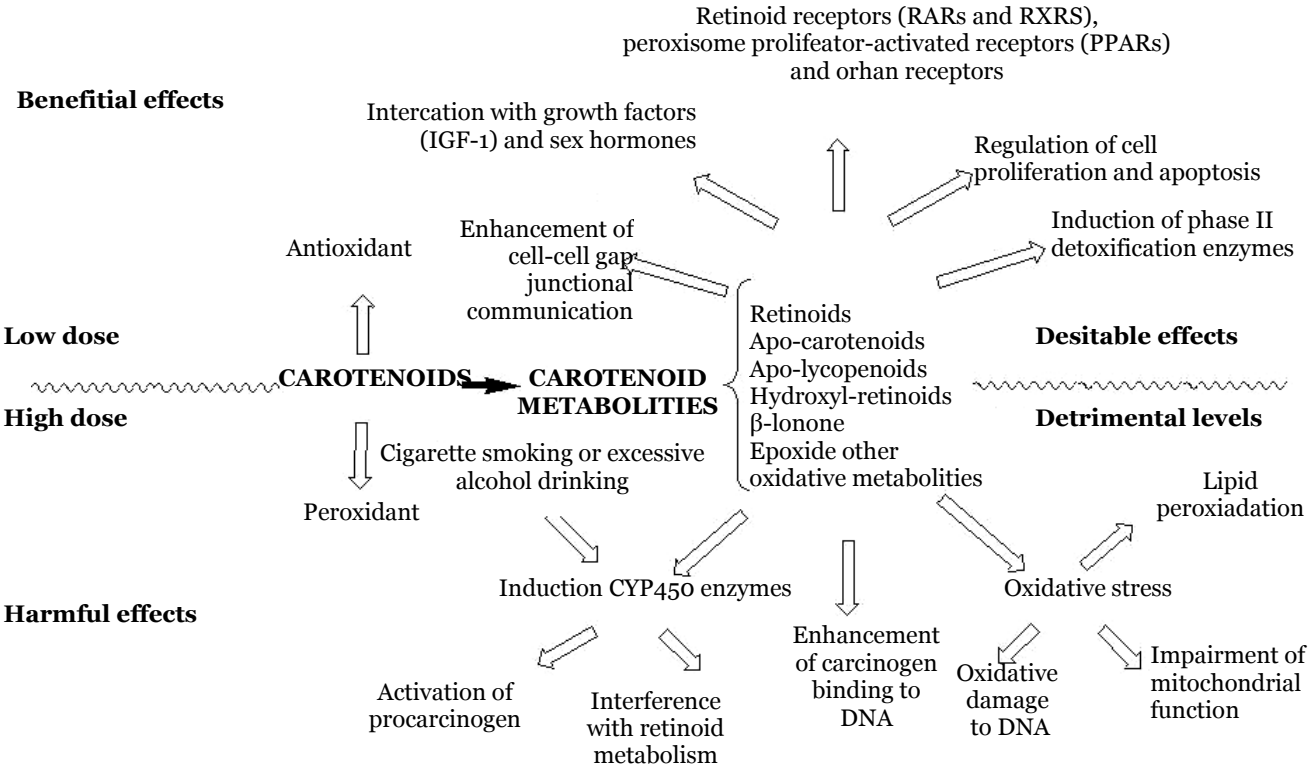


Fig. 3.7. Schematic illustration of possible mechanism(s) of carotenoid dose and oxidative metabolites on beneficial and detrimental effects to human health (Veeramachaneni and Wang, 2009).

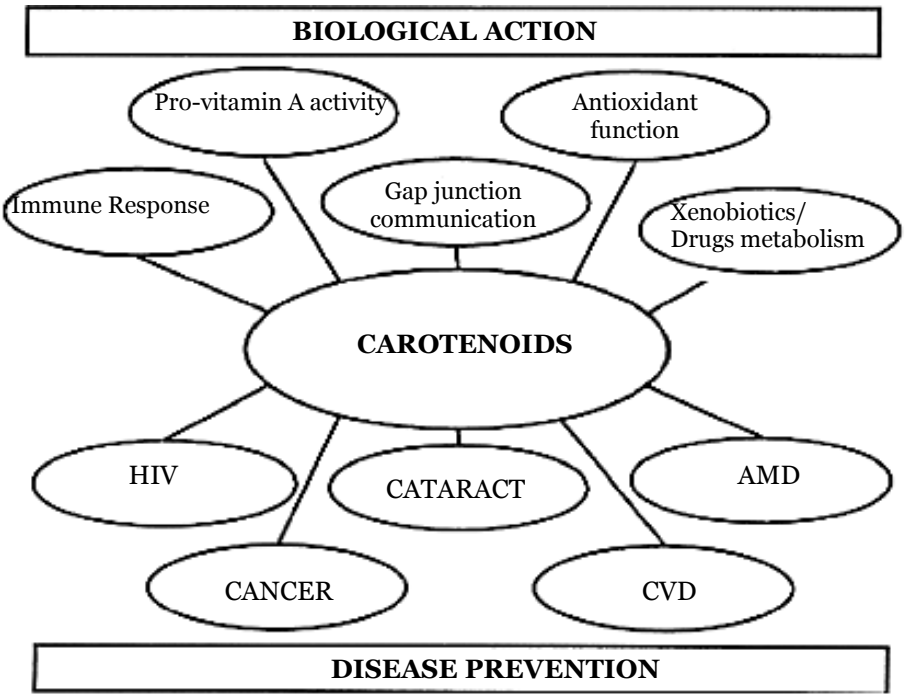


Fig. 3.8. Role of carotenoids in the prevention of chronic diseases (Rao and Rao, 2007).

Carotenoids in Capsicum fruits. The intensiv characteristic red colour of *Capsicum* fruits is principally due to the pigments of capsanthin and capsorubin that

represented 65-80% from the total colour. The yellow fraction mainly comprises of zeaxanthin, violaxanthin, antheraxanthin, *β*–cryptoxanthin, *β*–carotene and capsolutein, which are biosynthetic precursors of the red fraction. Of these pigments, only *β*–carotene and *β*–cryptoxanthin have vitamin A activity (Kim et al., 2011). Chemical structure of capsanthin, capsorubin and *β*–carotene are presented in Fig.3.9.

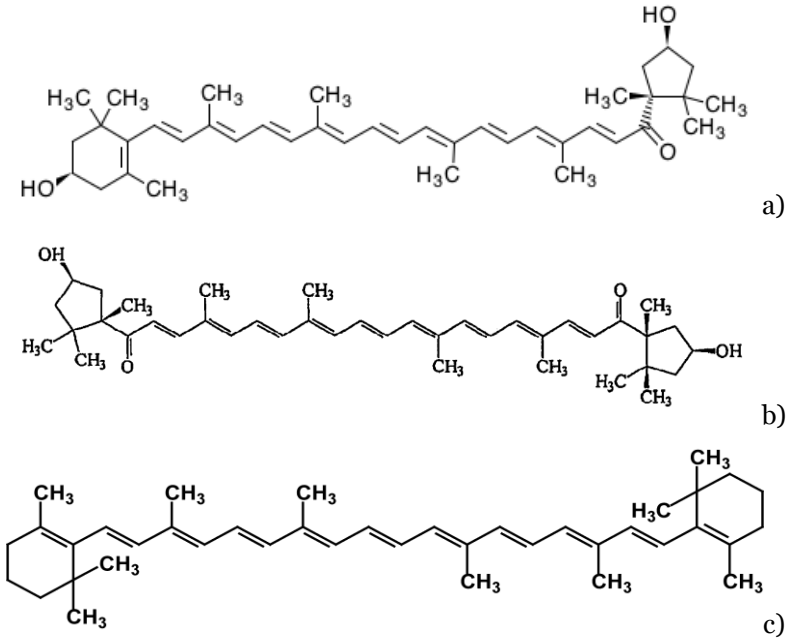


Fig. 3.9. Chemical structure of (a) capsanthin, (b) capsorubin and (c) *β*–carotene (Giuffrida et al., 2014).

The content of carotenoids in *Capsicum* fruits depend on numerous factors as genetic factors, variety of *Capsicum*, as well as the conditions of vegetation (environmental conditions, time of vegetation, soil conditions, degree of ripeness, country of origin, etc.) and processing . The total content of carotenoids in the dry fruits of red hot pepper ranges from 0.1 to 5% (Topus et al., 2007; Daood et al., 2014; Giuffrida et al., 2014).

The colour of *Capsicum* fruits can be determined by three aspects such as surface colour, colour extracted and carotenoid profile i.e. presence and quantity of carotenoids. The analytical methods that are currently used for determination of carotenoids in plant matrixes are: spectrophotometric methods, as well as chromatographic methods, thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC). The methods for carotenoid analysis usually require sampling and sample preparation, extraction,

saponification and washing, concentration or evaporation of solvent, identification and quantification (Giuffrida et al., 2014). The measurements for determining surface colour are made in order to determine the profile of the colour that is visible to the human eye by use of instrument colorimeter. The colorimeter is tristimulus i.e. three filtering device that uses red, green or blue filter which emulates the response of the human eye to light and colour. When colour is expressed in CIELAB system, the value of L* defines the brightness of the colour (0–dark, light–100), a * shows values that indicate red/green colour (ranging from -60 to + 6), and b * values indicate yellow/blue (-60 to +6). The interpretation of the obtained values of L*, a* and b* are performed using the scheme shown in Fig. 3.10. Chroma (C) describes the vividness or dullness of a colour, while H denotes hue angle(h°), an angular measurement (web 4, web 5).

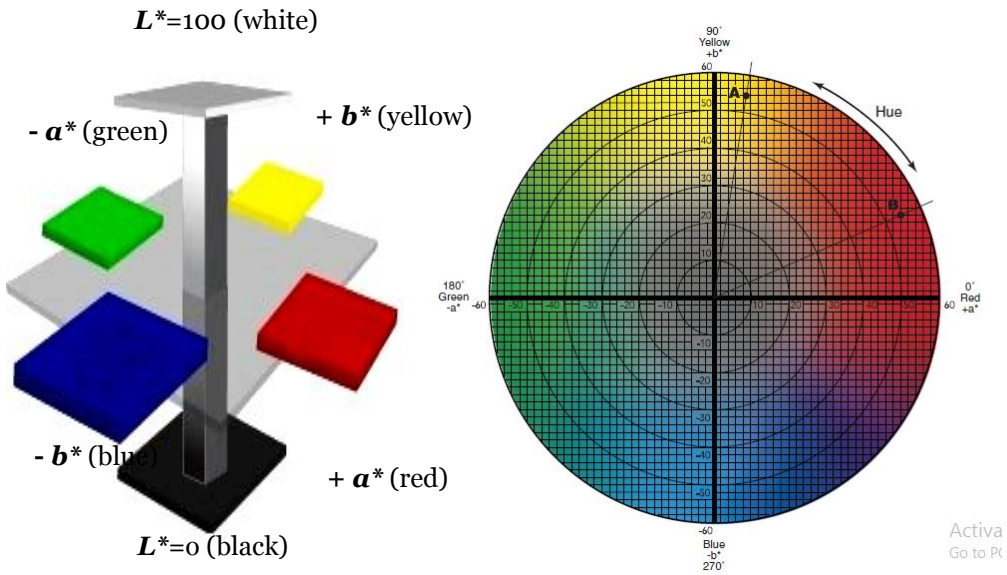


Fig. 3.10. CIELAB colour scheme (web 4, web 5).

The expressions for colour differences are ΔL*, Δa*, Δb* where Δ (delta) indicates difference. Given ΔL*, Δa*, Δb* the total difference or distance on the CIELAB diagram can be stated as a single value, known as $\Delta E^*_{ab} = [(\Delta L^{*2}) + (\Delta a^{*2}) + (\Delta b^{*2})]^{1/2}$. Differences ΔE* are interpreted as (web 5): normal difference non visible for the human eye (ΔE*,0–1), very small difference мноry, visible for one-third of the eye (ΔE*, 1–2), medium visible difference (ΔE*, 2–3,5), visible difference (ΔE*, 3,5–5) and very visible difference (ΔE*, < 5).

3.2. EXTRACTION OF RED HOT PEPPER

Extraction is a process in which one or more compounds are separated selectively from a liquid or solid mixture by means of a liquid immiscible solvent. The transfer of the compounds from the *feed* to the *solvent* is controlled by the solubility behavior of each component in the corresponding phase. Two phases result from the extraction step: *extract* and *raffinate*. Extracts can be defined as preparations which contain all the constituents which are soluble in the solvent used in making the extract. Afterwards in order to regenerate the solvent or to obtain pure compound, another separation step (e.g. distillation, crystallization etc.) is finally required. The term "*extraction*" is derived from the Latin word "*extrahere*"; "ex" specifies the direction, i. e. out of, and "*trahere*" describes the action, namely drawing or removing. Extraction is accordingly defined as the process of removing a substance or several substances from another substance. The process is extremely important in a wide range of technical applications, for instance biotechnology, the pharmaceutical and food industries as well as environmental protection. Extraction is a separating process which has the advantage of low energy consumption (Boonkird et al., 2008; Azmir et al., 2013).

The nature of the phases which are involved characterize the extraction process as solid-liquid extraction or liquid-liquid extraction. *Solid–liquid* extraction is a basic operation to separate one or more components contained in a solid phase by a liquid phase +or solvent. The components transferred from the solid to the liquid phase are called *solute*s. In the *liquid-luquid* extraction, one or more solute(s) are removed from one liquid phase (*diluent*) by transferring that/those the solute(s) to another liquid phase (*solven*t).

The following need to be carefully evaluated when optimizing the design and operation of the extraction processes: *solvent selection* (solvents differ in their extraction capabilities depending on their own and the solute's chemical structure); *operating conditions* (depending on the nature of the extraction process, the temperature, pH and residence time could have an effect on the yield and selectivity), *mode of operation* (extractors can be operated in crosscurrent or counter-current mode), *extractor type* (important factors to consider when selecting extractor types are the stage requirements, fluid properties and operational considerations).

3.2.1. Extraction with organic solvents

Generally, the most commonly employed and a preferred method for extraction of compounds present in plant matrices is the conventional solid-liquid extraction using organic solvents. In later studies, these conventional methods were improved, modified or rationalized by varying different operating parameters. In order to obtain bioactive

compounds from plants, the existing classical techniques are: (1) Soxhlet extraction, (2) Maceration and (3) Hydrodistillation (Azmir et al., 2013).

The solvent is the key factor to a successful separation of compounds by solid-liquid and liquid-liquid extraction. The several criteria are important: *selectivity* (the ability to remove and concentrate the solute from the other components in feed); *availability* (solvent in the extraction system can represent a significant capital investment), *immiscibility* (if the solvent is not immiscible with feed, reacovery of the solvent is required and it causes extra cost), *density difference* (small density difference of solvent and feed causes separation problems, lower capacity and larger equipment), physical properties: (a too viscous solvent inhibits mass transfer, the boiling point should be very different than that of the solute for recovery); toxicity (the solvent should be non-toxic for health), and ease of recovery (solvent recovery needs to be as complete and pure as possible for recycle and preventing pollution).

The extraction process of the raw materials with organic solvents has shown some disadvantages: solvents cannot be completely removed, high extraction temperature can cause extract destruction, solvent extraction is energy intensive, leads to problems of toxic waste disposal, gives a product that requires further purification, etc.

The red pepper oleoresins are produced by solvent extraction of dried, ground red pepper fruits, using a solvent-system with the lipophilic/hydrophilic characteristics and subsequent solvent-system removal. The solvents most commonly used for paprika oleoresin extraction are trichloroethylene, ethylacetate, acetone, propan-2- ol, methanol, ethanol and *n*-hexane. The oleoresin is further treated with polar solvent, methanol, in order to separate the pungent component from the colour component of the oleoresin (Boyadziev et al., 1999; Nazari et al., 2008; Bo et al., 2008; Boonkird et al., 2008; De Aguiar et al., 2014).

3.2.2. Supercritical fluids as solvents in high pressure processes

The high cost of organic solvents, increasingly stringent environmental regulations, and new requirements of ultra-pure and high added value products have increased the need for the development of new and clean technologies for the processing of food products. Extraction with supercritical fluids has attracted considerable attention in recent years as a promising alternative to conventional solvent extraction and mechanical pressing for extracting oils and other materials as it offers a number of advantages, including a lack of solvent residue and better retention of aromatic compounds (Sahena et al., 2009). The supercritical fluid extraction (SFE) method is very advantageous and environmentally friendly over other conventional either solvent or enzyme extraction methods for recovering natural oil.

The SFE is a separation technology that uses supercritical fluid (SCF) as the solvent. Every fluid is characterized by a critical point (CP), which is defined in terms of the critical temperature and critical pressure. The critical point represents the highest temperature and pressure at which the substance can exist as a vapour and liquid in equilibrium (Fig. 3.11.). SCF is defined as a substance above its critical temperature (T_C) and critical pressure (P_C). If the pressure is under the CP and the temperature below the CP, that fluids is named subcritical.

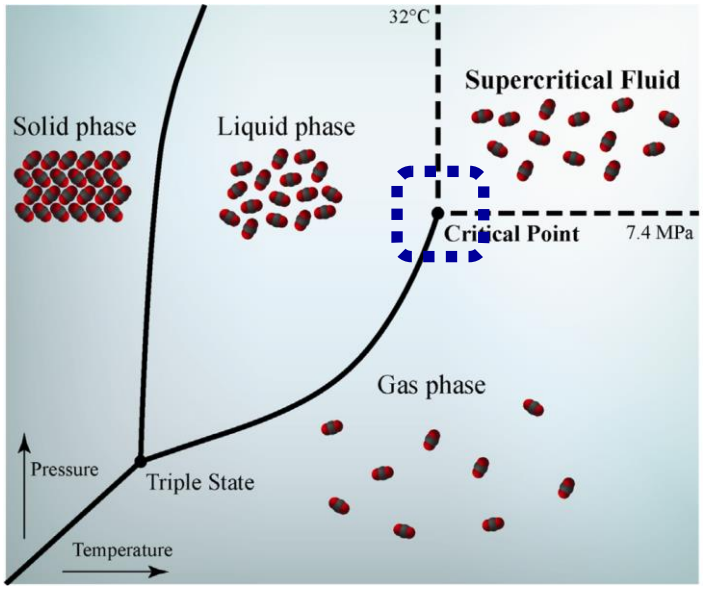


Fig. 3.11. Pressure-temperature phase diagram.

SFE provides several operational advantages over conventional extraction methods since it uses supercritical solvents (Askin, and Ötles, 2005; Sapkale et al., 2010), with different physicochemical properties such as *density*, *diffusivity*, *viscosity* and *dielectric constant*. Due to their low viscosity and relatively high diffusivity, supercritical fluids have enhanced transport properties than liquids, can diffuse easily through solid materials and can therefore give faster extraction rates. One of the main characteristics of a supercritical fluid is the possibility of modifying the density of the fluid by changing its pressure and/or temperature. Since density is related to solubility, by altering the extraction pressure, the solvent strength of the fluid can be modified. Other advantages, compared to other extraction techniques, are the use of solvents generally recognized as safe (GRAS), the higher efficiency of the extraction process in terms of increasing yields and lower extraction times, and the possibility of direct coupling with analytical chromatographic techniques such as gas chromatography (GC) or supercritical fluid chromatography (SFC).

Table 3.3. shows a comparison of some physical properties of a liquid, gas and fluid. The density change by increasing the temperature and pressure is shown in Fig. 3.12.

Table 3.3. Comparison of physical and transport properties of gases, liquids, and SCFs.

Property	Density (kg/m^3)	Viscosity (cP)	Diffusivity (mm^2 /s)
Gas	1	**0.01**	1-10
SCF	**100-800**	**0.05-0.1**	**0.01-0.1**
Liquid	**1000**	0.5-1.0	0.001

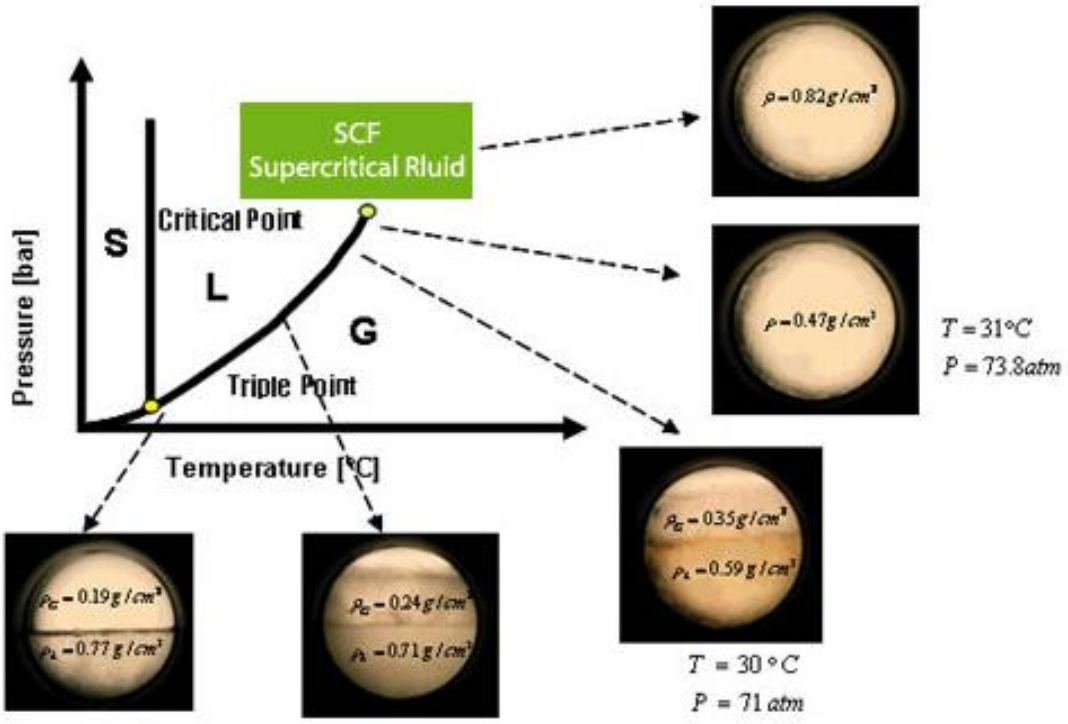


Fig. 3.12. Density of liquid and supercritical fluid.

Some solvents that are commonly used as supercritical fluids and their critical temperatures and pressures are shown in Table 3.4.

Table 3.4. Critical data of fluids.

Fluid	**Critical Temperature** (K)	**Critical Pressure** (bar)
Carbon dioxide	**304.1**	**73.8**
Ethane	305.4	48.8
Ethylene	282.4	50.4
Propane	**369.8**	**42.5**
Propylene	364.9	46.0
Water	**647.3**	**221.2**
Cyclohexane	553.5	40.7
n-Pentane	469.7	33.7
Toluene	591.8	41.0

Several supercritical fluids have been studied, including propane and water, but the most widely used is supercritical CO_2. Supercritical carbon dioxide application advantages are:

- CO2 is supercritical above 31.1 °C and 73.8 MPa, which makes it an ideal solvent for extracting thermally sensitive materials;
- 3 S comply with environmental standards (Reduce-energy conservation and reduce pollution; Reuse-repeated, repeated use; Recycle-sustainable resources, recycling);
- Carbon dioxide is in fact industrial **by-products**, such as the fermentation industry, the petrochemical industry in the process will have a high concentration of carbon dioxide;
- sterile and can inhibit bacterial growth
- non-toxic, safe, no explosive, non-corrosive, chemical stability, non-toxic; does not need to meet fire, explosion or anti-corrosion of special protective equipment.

  95% more CO2 recovery , very easily and can be completely separate and solute, the solute and solvent that is easy to accept, without question residual reserves.

The basic SCF plant (Fig. 3.13) consist of an extraction vessel, where the raw material is charged. The liquefied CO2 is heated and enters the extraction vessel from the bottom, SCF at the exit of the extractor flows through a depressurization valve to a separator in which, due to the lower pressure, the extracts are released from the gaseous medium and collected. In order to fractionate the obtained extract more separators can be added in which by setting different temperature and pressure, the fractions of different compositions are separated, Fig. 3.14.

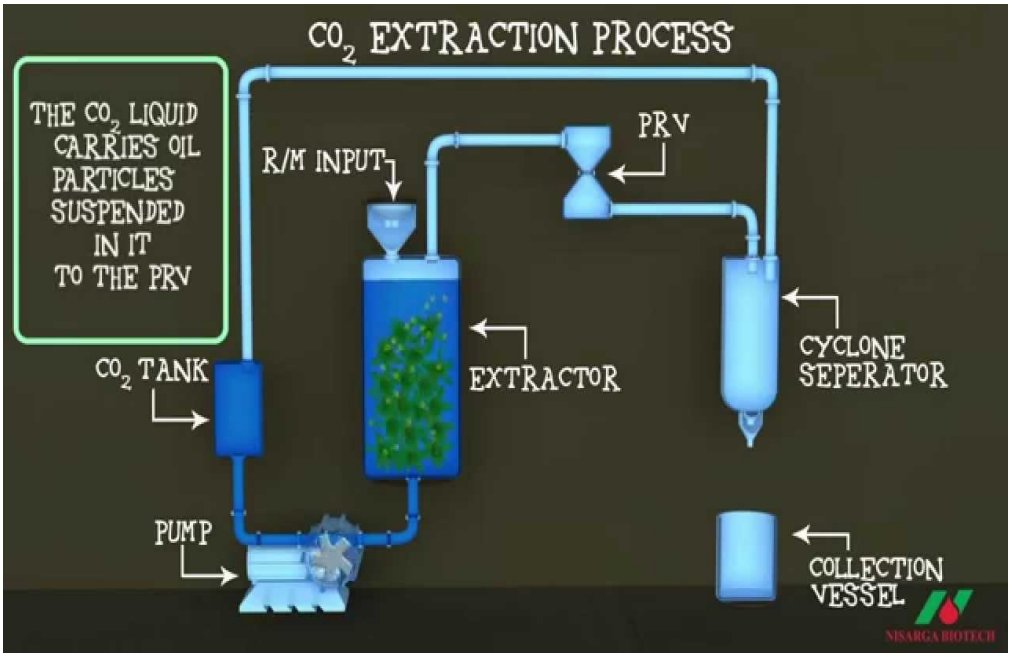


Fig. 3.13. Schematic diagram of supercritical apparatus.

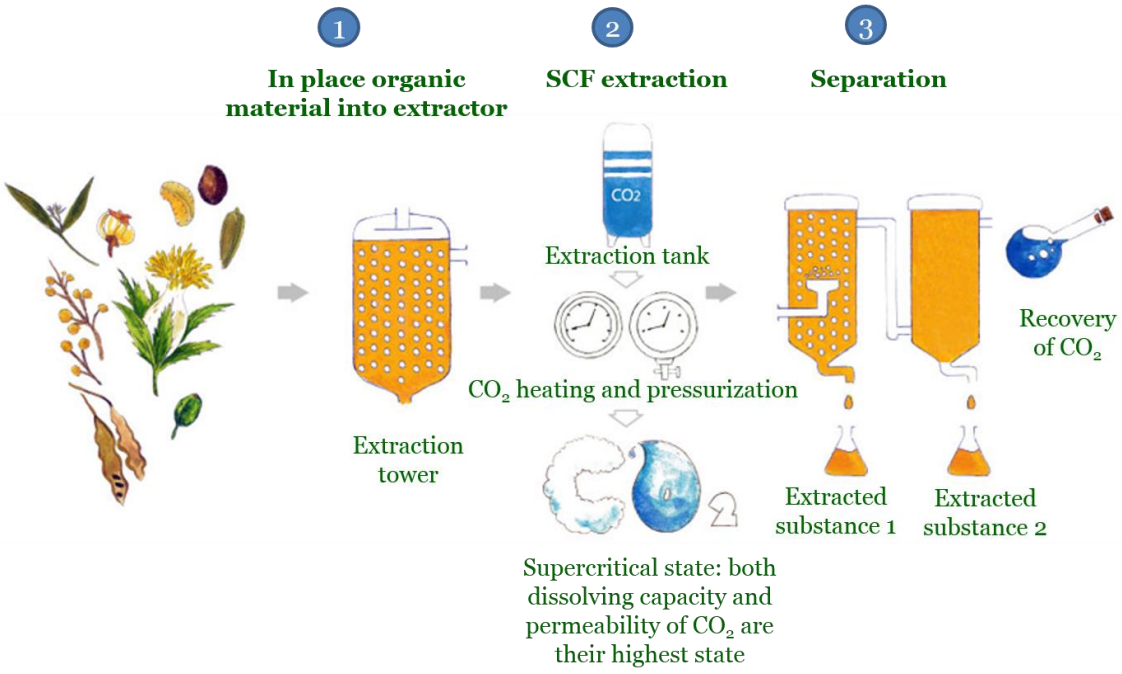


Fig. 3.14. Schematic diagram of extraction with SCO_2 and two-step separation of extract.

The knowledge of the effects of the operational variables and the study of supercritical extraction curves allow the establishment of the extractor volume and solvent flow rate

(QCO2). According to Da Silva et al. (2016), the overall extraction curves (OEC), are clearly divided into three periods (Fig. 3.15) controlled by different mass transfer mechanisms: (1) Constant Extraction Rate (CER) period, where the external surface of the particles is covered with solute (easily accessible solute) and the convection is the dominant mechanism of mass transfer; (2) Falling Extraction Rate (FER) period, where failures in the external surface oil layer appear and the diffusion mechanism starts, operating combined with convection; (3) Low Extraction Rate (LER) or Diffusion-Controlled (DC) period, where the external layer of oil practically disappeared and the mass transfer occurs mainly by diffusion inside the solid particles.

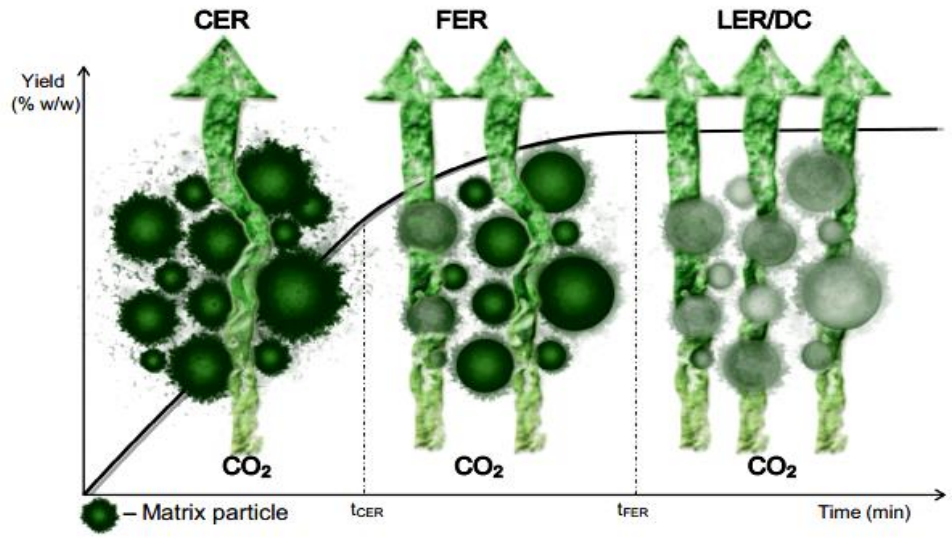


Fig. 3.15. The three different extraction periods and its extraction curve (Da Silva et al., 2016).

At extraction of pericarp of red hot pepper with supercritical fluids, the influence of the pressure, temperature and flow rate of was studied (Duarte et al., 2004; Perva-Uzunalić et al., 2004; De Aguiar et al., 2015; Santos et al., 2015)

4. EXPERIMENTAL WORK

4.1. MATERIAL

4.1.1. Red hot pepper

Pepper, *Capsicum annuum* L., was grown in the locality of Markova Češma, Prilep (geographical location: +41°21'36" N latitude, +21°33'36" E longitude and 640 m altitude), Republic of Macedonia, in the year 2014. The basic treatment of the soil was performed by deep ploughing up to 40–50 cm. In order to make it soft prior to sowing, the soil was ploughed by turning over. Prior to the autumn ploughing and planting out of the seedlings, 20–30 t/ha manure and 2 t/ha macronutrients, i.e. nitrogen, phosphorus and potassium (NPK), 10:30:20, respectively, were added. The nitrogen fertilizer was added three times, first with 300 kg/ha calcium ammonium nitrate (CAN) 25–30 days after planting, the second time 20–25 days after the first addition, and the third time in the stage of intensive growth of fruits, in combination CAN (27% calcium ammonium nitrate) and NPK (10:30:20), 200 kg/ha each. The average monthly air temperature ranged between 11.7 °C and 19.1 °C during the vegetation of the pepper from May to September 2014. The total amount of precipitation in the period of vegetation was 180 mm/m^2. Irrigation was applied by a drip irrigation system (300 m^3 water/ha) from the hydrosystem in Prilep. The seed of this pepper variety was from the manufacturer's own production. For many years, this variety pepper has been grown in the area Markova Češma and has been adapted to outdoor growing conditions. The vegetation period of the variety pepper was 70 days. The fruits are usually harvested when they are full grown and mature, 10.5 to 11.5 cm long, firm and deep red. They were harvested manually, and dried in a dry and ventilated place. After drying, they were cut manually longitudinally with a knife, and pericarp was separated from the seed and placenta of the dry fruitsand ground using a Retsch ZM1 mill (Germany), with a 0.5 mm sieve. The pepper samples were placed in dark glass bottles and stored at 4 °C in a refrigerator. The determination of the pepper variety was done at the Faculty of Agricultural Sciences and Food, at Ss. Cyril and Methodius University in Skopje, R. Macedonia. The pepper belongs to the species *Capsicum annuum* L., ssp. *Microcarpum longum conoides*, convar. Horgoshka. The fruit size characteristics were determined by measuring the length from the base to the apex of the fruit without the pedicle and the width at the widest part of the fruit. The weight fraction of the pericarp, placenta, seed and stem was determined by weighing the fruit part with 0.0001 g accuracy. The length and weight, as well as the weight fraction of the separated pericarp, seed, placenta and stem of the fruit were determined as an average value of ten measurements. In general, the fruits of pepper *Capsicum annuum* L., ssp. *microcarpum*

longum conoides, convar. Horgoska were oblong in form, with a typical deep red colour, average length of 10.80 ± 0.72 cm and average width of 3.26 ± 0.23 cm. The average weight per separated pericarp, seed, placenta and stalk from the dried fruits of red hot pepper was 4.43 ± 0.02 g, 2.37 ± 0.08 g, 1.57 ± 0.21 g and 0.49 ± 0.14 g, respectively. The view of red hot pepper is presented at Fig. 4.1.

Fig. 4.1. Red hot pepper: (a) whole fruits, (b) pericarp, (c) placenta, (d) seed and (e) stalk.

4.1.2. Chemicals

Reagent grade chemicals used for plant material characterization were purchased from Alkaloid AD (R. Macedonia) and Merck (Germany).

For the extraction of plant material and determination of carotenoids and capsaicinoids analytical grade ethanol, methanol, acetone, *n*-hexane and and petroleum ether 40–60 °C supplied from Merck (Germany) were used. In the extraction of plant materials at supercritical conditions, propane (>99.5% purity, Linde Plin, Cejle, Slovenia) and carbon dioxide (>99.5% purity, Messer, Ruše, Slovenia) were used.

The capsaicin standard (65% pure) was supplied by Fluka (Switzerland) and the dihydrocapsaicin standard (90% pure) was from Sigma-Aldrich Inc. (USA). Standards: 1–propanol, hexyl hexanoate, hexyl isopentanoate, heptyl butanoate, benzyl benzoate, hexyl isobutanoate, hexyl 2–methyl butanoate, α–ionone, β–ionone, and tetradecanoic acid were purchased from Fluka (Switzerland), with a purity >98%. Headspace vials (22 mL volume) were supplied from Perkin-Elmer (USA).

For the determination of antioxidative capacity of the lipid soluble compounds were used analytical grade methanol (Merck, Germany) and reagents: reaction buffer, photo sensitizer and detection reagent, and calibration standards purchased from Analytik Jena AG (Germany).

In the preparation of edible films sodium alginate (Sigma-Aldrich Company Ltd, USA.), Tween 80 (Merck, Germany) and glycerol (Merck, Germany) were used. Identification of the microorganism was done on the *Sabouraud*–dextrose and *Nutrient* agar (Sigma-Aldrich Chemie GmbH, Germany)

Nanoemuslion formulations were developed using surfactants Span 80, Tween 80 and Tween 20 purchased from Merck (Germany), ultrahigh purity Mili Q water (PURELAB classic system, 18.2 MΩ-cm, ELGA, USA), gelatin and lecithin (P.I.C. Co, R, Macedonia).

At application of red hot pepper extract for control of green peach aphid on tobacco, non-ionic surfactant Brij 35 (Merck, Germany) and commercial insecticide Confidor SL 200 (Bayer CropScience, Germany) were used.

4.2. EXTRACTION OF RED HOT PEPPER

4.2.1. Conventional methods for extraction

4.2.1.1. Maceration in thermostatic water bath

Extraction was performed in thermostatic water bath with ethanol as solvent. The values of the temperature, time of extraction and solid to liquid ratio are presented in Table 4.1. A 1 g sample (0.0001 g accurately weight) was used in the extraction process. After extraction for selected time and at maintained temperature, the solvent was removed under vacuum (rotary vacuum evaporator, type Devarot, Slovenia, 35°C, atm. pressure). Solvent traces were discharged by drying the sample at 40°C, 105 mPa (vacuum drier, Heraeus Vacutherm VT 6025, Langenselbold, Germany). Each extraction procedure was performed in duplicate under the same operating conditions. Obtained extracts were cooled in a desiccator and weighting. The steps of drying, cooling and weighting were repeated until the difference between two consecutive weights was smaller than 2 mg.

Experimental design. Central composite design with three variables and two levels consisted of 13 runs with two variables and five replicates of the central point for the estimation of pure error was selected. The coded and uncoded values of independent variables used in the central composite design are listed in Table 4.1.

Table 4.1. Central composite design: coded levels of independen variables.

Level and interval of variation	**Time** - X_1 (min)	**Temperature** - X_2 (^oC)	**Solid/liquid ratio – X_3** (g/cm^3)
$\mathbf{X_i = +1}$ (max level)	300	60	0.1
$\mathbf{X_i = -1}$ (min level)	60	20	0.025
$\mathbf{X_i = 0}$ $\mathbf{X_{io} = [X_{(i = +1)} + X_{(i = -1)}]/2}$ (central ниво)	180	40	0.04
$\mathbf{(\Delta X_i) = [X_{(i = +1)} - X_{(i = -1)}]/2}$ (interval of variation)	120	20	0.063

4.2.1.2. Ultrasound extraction

In ultrasound water bath type DC150H (R. Macedonia) by changing the operational parameters: temperature, time and solid and liquid phase using ethanol and *n*-hexane as solvents, extraction of red hot pepper was performed (Fig. 4.2). The procedure of after extraction was the same as given in chapter 4.3.1.1. Each experiment was performed in duplicate under the same operating conditions.

Fig. 4.2. Ultrasound extraction of red hot pepper.

Experimental design. Full factorial design with three variables and two levels consisted of 16 runs was selected. The coded and uncoded values of independent variables used in the central composite design are listed in Table 4.2.

Table 4.2. Full factorial design 2^3: coded levels of independen variables.

Level and interval of variation	**Time - X_1** (min)		**Temperature – X_2** (°C)		**Solid/liquid ratio – X_3** (g/cm³)	
	E	H	E	H	E	H
$X_i = +1$ (max level)	30	30	70	60	0.1	0.1
$X_i = -1$ (min level)	5	5	20	20	0.05	0.05
$X_i = 0$ **$X_{io} = [X_{(i=+1)} + X_{(i=-1)}]/2$** (central ниво)	17.5	17.5	45	40	0,04	0,04
$(\Delta X_i) = [X_{(i=+1)} - X_{(i=-1)}]/2$ (interval of variation)	12.5	12.5	25	20	0,067	0,067

E – ethanol; H – *n*-hexane

4.2.1.3. Soxhlet extraction

Soxhlet procedure no. 920.85 (AOAC, 2006) was used for the red hot sample extraction. A 5 g of sample (0.0001 g accurately weighed) was extracted in the presence of 10 boiling glass regulators with 200 mL solvent (*n*-hexane, ethanol or petroleum ether 40–60°C), Fig 4.3a. After 5 h extraction, the solvent was removed from the extract (Fig. 4.3b) by using rotary vacuum evaporator type Devarot (Slovenia) at 40 °C and 200 mPa). The solvent traces were removed by vacuum drying (Heraeus Vacutherm VT 6025, Germany) at 40 °C and 105 mPa followed by cooling in a desiccator and weighing. The steps of drying, cooling and weighing were repeated until the difference between two consecutive weights was

smaller than 2 mg. The yield of obtained extract was calculated based on dry matter (DM) weight of sample used.


a)

b)

Fig. 4.3. Extraction of red hot pepper by *Soxhlet* procedure (a), and obtained extract (b).

4.2.2. Supercritical fluid extraction

4.2.2.1. Supercritical fluid extraction with carbon dioxide

Semi batch experiments were performed on an apparatus manufactured by the company UHDE-GmbH-Hagen (Fig. 4.4 and 4.5) .Technical data of the plant are: volume of the semi batch operated extractors V_{B1}=4 L and V_{B2}=0.5 L; maximal working pressure and temperature are 500 Pa and 120 ^{o}C. To this equipment two continuous operated countercurrent columns were built. Column K1 of active height of 3 m has a diameter of 52 mm, maximal working pressure and temperature are 300 Pa and 100 ^{o}C. Column K2 is 2.5 m high and has a diameter of 25 mm. Maximal working pressure and temperature are 500 Pa and 100 ^{o}C. The flow rate of CO_2 can be varied between 5 and 33 kg/h, while the flow rate of liquid phase between 0.05 and 20 kg/h. CO_2 flows through the filter F1 and condenser C1 where it is cooled and condensed into a reservoir D1. From the reservoir, it flows through a heat exchanger W3 where it is additionally cooled to 0 ^{o}C to the high pressure membrane pump P1. The pump P1 brings CO_2 on the operating pressure and pumps the fluid through the heat exchanger where it is heated to the operating temperature to the extractor B1 or B2 or in the one of the columns K1 or K2. In the case of semi batch process, one of the extractors B1 or B2, which are equipped with the heating jackets, is preliminary loaded with an amount of raw material. The solute is solubilized in a solvent (CO_2). The separation is carried out in a separator S1, where the two phase area is attained with the help of the expansion valve and

with heating in the heat exchanger W2. The solute can be taken with help of the valve at the bottom of separator. The remained CO_2 flows through a filter F2 to the condenser and reservoir from where it is recycled to the process. In the extraction of red hot pepper with supercritical carbon dioxide, on the yield of extract and content of capsaicinodis and total colour compounds, was investigated the influence of the particle size of raw material (0.25 mm, 0.5 and 1 mm), temperature (40°C) and pressure (400 bar). The extraction kinetic was followed by taking samples on every 15 minutes in the first hour, and on every hour to constant extract weight achieving. Content of capsaicinoids and total colour compound were determined.

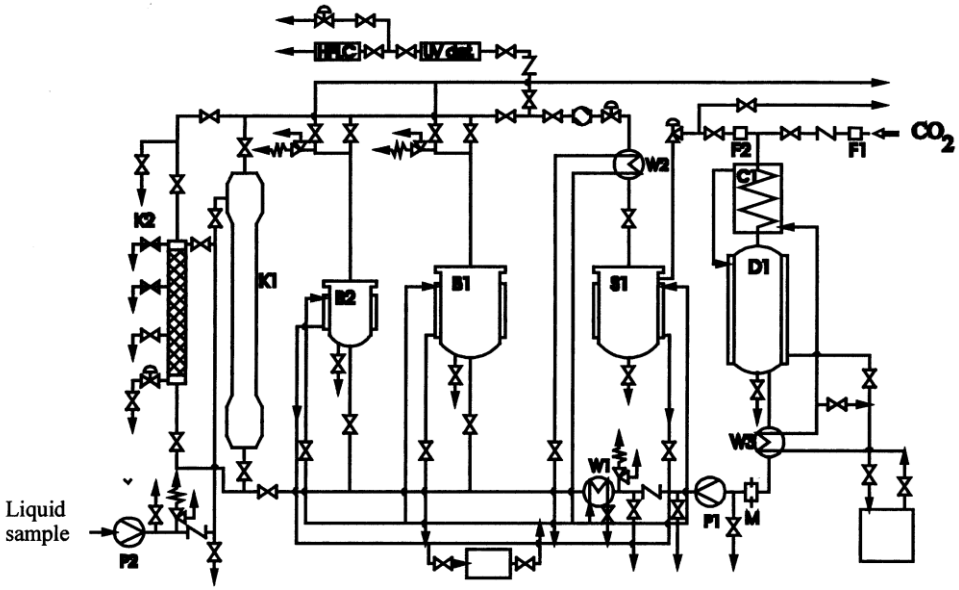


Fig. 4.4. Apparatus for supercritical extraction. B, autoclave; C, condenser; D, reservoir; F,filter; K, column; M, flow meter; P, pump; S, separator; W, heat exchanger (Škerget and Knez, 2001).

Fig. 4.5. High pressure extraction apparatus.

4.2.2.2. Supercritical fluid extraction with propane

The extraction with dense propane was performed on a dynamic flow semi continuous apparatus (Fig 4.6) which was home build for production of natural extracts with flammable gases at a maximum pressure of 500 Pa and a temperature of 100 ^{0}C. Schematic design of extraction system is presented in Fig. 4.7. It is equipped with an extraction vessel with volume 60 mL, 60-200 bar working pressure and 20-100 $^{\circ}$C temperature. The temperature in the water bath was regulated and maintained at constant level (± 0.5 ^{0}C, LAUDA DR. R. OBSER GmbH & Co. KG, Lauda Königshofen, Germany). The separator (sample trap) was with 0.1 L volume and 10 mm i.d. The high pressure ISCO syringe pump was model 260D (Lincoln, Nebrasca, P_{max}=350 Pa). The solvent flow rate was measured with a flow-meter (ELSTER HANDEL GmbH, Mainz, Germany).

a) b) c)

d) e) f)

Fig. 4.6. System for extraction with dense propane (a): high pressure pump (b), regulator and temperature indicator (c) gas bottle (d), rotameter (e) and pressure indicator (f).

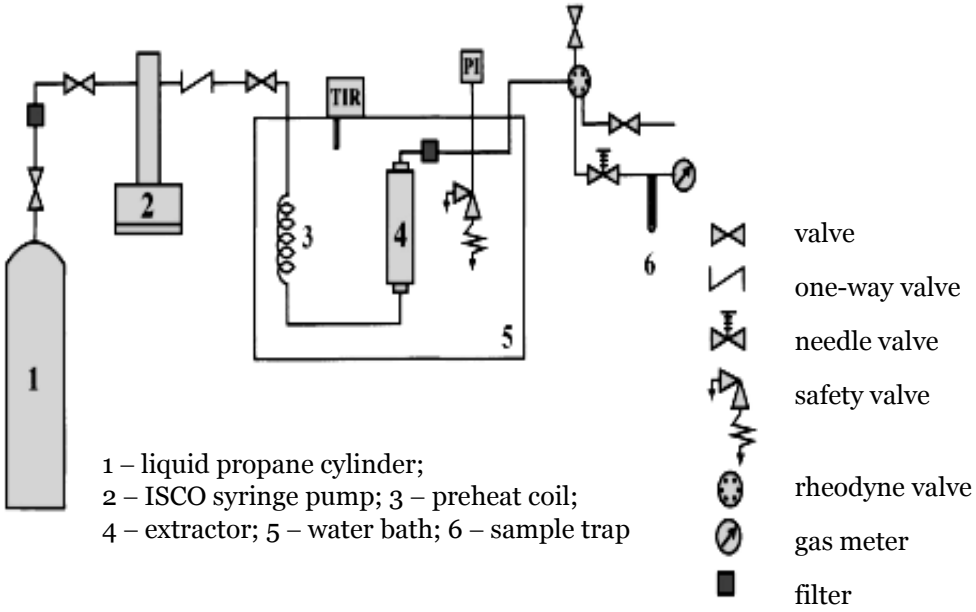


Fig. 4.7. Diagram flow of the extraction equipment with dense propane (Hadolin et al., 2001).

Approximately 20 ± 0.0001 g of ground material was charged into the extractor (V=60 ml). The temperature in the water bath was regulated and maintained at constant level (30 ± 0.5 °C). The apparatus was purged first with nitrogen and later with the propane used for extraction. In the next step, liquefied propane with 2 mL/min flow rate was continuously pumped with a high pressure pump through the preheating coil and over the bed of sample in the extractor. The extract was separated from the dense propane by pressure difference between autoclave extractor and environment. The pressure and temperature in the separator were set at 1 Pa and 25°C. Different conditions: pressure (20, 50 and 100 Pa), sample particle size (0.25, 0.5 and 1 mm), temperature of 30 °C, 2 mL/min flow rate of dense propane were used at extraction of red hot pepper samples. The extraction was performed until the difference between two consecutive weights of the extract was smaller than 0.1 mg (around 3h). The extract was kept at 4 °C to further analysis.

4.3. PREPARATION OF FORMULATIONS FROM RED HOT PEPPER

4.3.1. Edible film formulation

The extract obtained by SCO_2 extraction of red hot pepper seed were introduced in *Na* – alginate edible coatings. The *Na*–alginate solution (2% w/v) was prepared by dissolving 2 g *Na*–alginate into 100 mL distillated water with stirring at magnetic stirrer (Tehtnica Zeleznik, Slovenia) at 50 °C for 40 min. After the complete dissolution of the *Na*– alginate, Tween 80 (2 mg), glycerol (2 mg) and seed extract in quantity of 10, 20, 30, 50 and 100 mg

were added, and the solution was stirred for another 30 min. The prepared solution was transferred to a square plastic (polypropylene) casting container (12 x 12 cm) ensuring that all air bubbles were removed from the viscous medium before. The films were dried at room temperature (20 ± 2 °C) for 48 h. The dried alginate films were peeled off from the container before characterization. The edible films were characterized for water vapor permeability, optical properties, texture and antimicrobial activity.

4.3.2. Nanoemulsion formulation

Nanoemulsion formulation were developed from red hot pepper seed extract obtained by SCO_2 extraction using spontaneous emulsification method and high energy method.

4.3.2.1. Spontaneous emulsification method

Spontaneous emulsification is one of the low energy method used in the preparation of oil-in-water nanoemulsions (Hasani et al., 2015). The seed oleoresin of red hot pepper obtained by SCFE at 400 bar and 40 ºC at UHDE apparatus were mixed with different surfactant mixtures in ratio of 1:1 w/w. The estimated value of hydrophilic lipophilic balance (HLB) for the of surfactant mixture Span 80 and Tween 80 was 10.7, while for Span 80 and Tween 20 was 11.32. Acetone (40 mL) in quantity in the mixture of seed oil and surfactant mixture was added. After treatment on ultrasound bath (Frutsch laborette 17.002, 220 V, 35 kHz, Germany) for 15 min, 10 mL aqueous phase with the flow rate of 1 mL/min was injected into organic phase by stirring at magnetic stirrer (Rotamix 604 MM, Domel, Slovenia) at 700-800 °/min for 30 min, to reach phase system equilibrium. The mixture was than evaporated to BUCHI Rotavapor R-114 (Switzerland) at 40° C and Buchi vacuum controller (Switzerland) B-721 at 250 bar, in duration of 45 min up to 1 h. The nanoemulsions were stored at 4 and 25 ºC.

The droplet size and the droplet size distribution, the zeta potential (droplet surface charge) and the polydispersity index (PDI) as a measure of the size variation were determined. The stability of nanoemulsions was evaluated at 24h and 10 days after preparation.

4.3.2.2. High energy method

Nanoemulsions of oil extracted from red hot pepper seed were developed using high energy method according the procedure described by Xue (2015). In the gelatin (6 g) dissolved in MiliQ ultra-pure water (90 mL), different amounts of lecithin (0.5 – 1g) was added into the

gelatin solution, followed by treatment at ultrasound bath (Frutsch laborette 17.002, 220 V, 35 kHz, Germany) for 1 h at room temperature (20 ± 2 °C). The seed oil solution was prepared by dissolving 1 g into 10 mL polyethylene glycol (PG). The gelatin/lecithin sample and seed oil solution were be mixed and emulsified using a high pressure homogenizer type APV - 2000 (SPX Flow Technology, United Kingdom, PH = 300/100 bar; n = 5). In the emulsion samples with seed oil two phases were separated. Sensory the emulsion appears to be stable. The distribution of drops was measured by Mastersizer 2000 apparatus, Fritarom, Etol, doo. The samples tail was spotted as a phenomenon (>100μm). The cause for this phenomena is probably too high pressure of the homogenization method. Also the samples were dissolved with water (1:50 w/w) and measured droplet size, distribution as well as zeta-potential using a Zetasizer Nano ZS device (Malvern Instruments Ltd, United Kingdom). The measurements were conducted at 25 °C. Each sample was measured with Zetasizer Software Version 7.11, in three successive runs and in each run the sample was scanned ten to 100 times for the potential. The gelatin/lecithin samples and seed oil solution were also mixed and emulsified using a high pleasure homogenizer Polytron PT1200 (12-18 V, max power 100 W, 5.000-25.000 RPM, Kinematika AG, Switzerland) with 16.000 °/min (RTM). Time of rotation for each sample was 5 minutes.

4.3.3. Biopesticide formulation

The extracts obtained from the separated anatomical parts of the fruit of red hot pepper, pericarp, placenta, seed and stalk were dissolved in redistilled water (0,02% w/v), of ultrasonic bath (Clifton, UK) for 3 min. To obtain a stable emulsion in the seed extract 25 mg of non-ionic surfactant Brij 35 (Merck, Germany) was added. Efficiency of hot pepper extracts in controlling and destroying the green aphid (*Myzus persicae* Sulz) on tobacco plants was compared with commercial insecticide Confidor SL 200 (Bayer CropScience, Germany) with 0.02% concentration of the active ingredient imidacloprid.

4.4. ANALYTICAL METHODS

4.4.1. Plant material composition analysis

The nutritional chemical composition of the red hot pepper was determined through standard procedures given by AOAC (2006): moisture content involved drying at 105 °C until constant mass (925.10), total proteins based on the nitrogen content (N x 6.25) determined by using the Kjeldahl method (978.04), crude fats by extracting with *n*-hexane or petroleum

ether (40–60 °C) using Soxhlet method (920.85), ash by mineralization at 900 °C (923.03), macro and microelements by atomic absorption spectroscopy (985.29), and crude fiber with gravimetric procedure (985.29). Total and reductive sugars were determined using the *Bertrand* method (Trajković et al., 1983). Energy content was calculated according to the food – energy conversion factors (FAO, 2003) by following equation: energy (kcal) = 2.44 (g proteins) + 3.37 (g total carbohydrates) + 8.37 (g fats).

4.4.2. Surface colour determination

Colour parameters, L* (lightness–darkness), a* (red–green), b* (yellow–blue), C (chroma), and Hue angle (Ho) of red hot pepper were measured using Dr. Lange spectra colorimeter (Chelmsford, UK). The samples were placed into a 1 cm cell. L*, a*, b*, C, and Ho values were determined using Illuminant D65 and 10° observer angle. The standardized values for a white plate were L*=95.93, a*=–0.19, and b*=3.12.

4.4.3. Volatile compound analysis

Types and concentrations of volatile flavor compounds were determined using headspace gas chromatography system (HS-GC), *Shimadzu GC 2010-Plus* (Japan), equipped with a flame ionization detector, and capillary column ZB-5 (30 m x 0.25 mm i.d., 0.25 μm film thickness, Phenomenex, USA). *Chromatographic conditions*: FID detector temperature 290 °C; Helium carrier gas, flow rate 1.0 mL/min. *Column temperature program*: initial 40 °C, rate 5 °C/min to 280 °C, held at 280 °C for 5.0 min, split ratio =1:40. *Shimadzu GC 2010-Plus Headspace* (HS-20) system, Oven: 150 °C; Needle: 150 °C; Transfer line: 150 °C; Sample equilibration: 20.0 min at 100 °C; Pressurization: 3.0 min; Sample transfer time: 0.5 min; Withdraw needle: 1.0 min. Identification of the volatiles was based on comparison of GC retention time of reference standards (RS). The volatile compounds determination was done in duplicate for each stalk sample. A typical chromatogram of volatiles is shown in Fig. 2.

The samples were also analyzed using headspace gas chromatography mass spectrometry system (HS-GC-MS), Shimadzu GCMS-QP2010 Ultra gas chromatograph (Japan), Capillary column ZB-5MS (30 m x 0.25 mm i.d., 0.25 m film thickness (Phenomenex, USA). Chromatographic conditions: Helium carrier gas, flow rate 1.0 mL/min; Column temperature program: initial 40°C, hold for 1 min, rate 5°C/min to 280 °C, hold 5.0

min; Split ratio =1:40. Shimadzu auto sampler AOC-5000 plus HS, Incubation: 50 ºC, 78 ºC, 100 ºC and 150 ºC. Needle: 150 ºC; Sample equilibration: 20 min at incubation temperature; Identification of the volatiles was based on comparison of GC-MS NIST library according to mass spectra.

A typical chromatogram of volatiles analyzed by HS-GC and HS-GS-MS is given in Fig. 4.8.

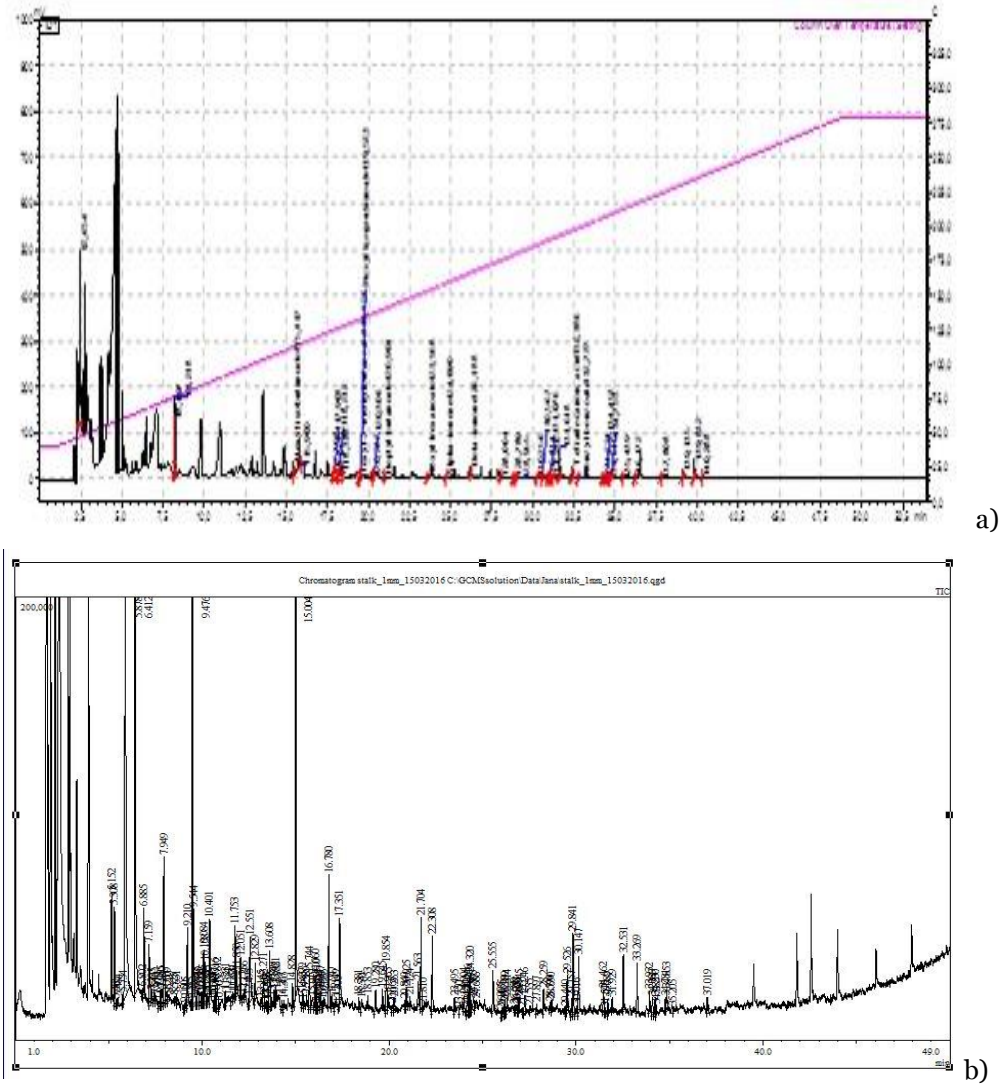


Fig. 4.8. Chromatogram of volatiles of red hot pepper stalk with 1 mm particle size: (a) HS-GC and (b) HS-GC-MS method.

4.4.4. Determination of capsaicinoids

4.4.4.1. Determination of capsaicinoids by gas chromatography (GC)

A Perkin Elmer Clarus 500 GC gas chromatograph (USA) equipped with a flame ionization detector (FID) and Elite 1 capillary column (30 m × 0.53 mm i.d., 1 μm film thickness, Perkin Elmer, USA) was used for capsaicinoid analysis. 1 μl of plant material extract was injected at 260 °C. The oven temperature program was as follows: 225 °C at the start, 25 °C/min to 275 °C (1.5 min hold), 10 °C/min to 325 °C. Nitrogen carrier gas was applied. The quantity of capsaicin and dihydrocapsaicin were determined from the calibration curves of capsaicin and dihydrocapsaicin. The capsaicinoid content in the plant materials expressed in mg/g dry matter weight was estimated as the sum of capsaicin and dihydrocapsaicin. Scoville heat values (SHU) were calculated from the capsaicinoid content given in mg per kg sample dry mass by multiplying by a factor of 16.1 (Todd et al., 1977). Fig. 4.9 (a, b and c) shows the gas chromatograms of the extract from the pericarp, seed and placenta of red hot pepper, respectively. The first, highest peak shows capsaicin (1, *t*R=5.80 min) followed by the peak of dihydrocapsaicin (2, *t*R =5.95 min).

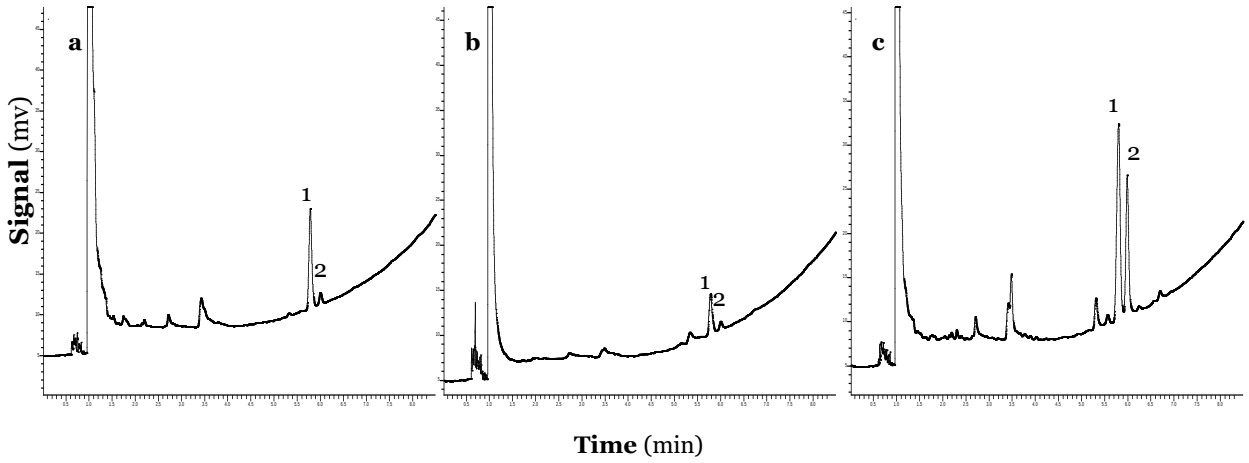


Fig. 4.9. Gas chromatograms of the extract of the (a) pericarp, (b) seed and (c) placenta of red hot pepper (1, capsaicin; 2, dihydrocapsaicin).

4.4.4.2. Determination of capsaicinoids by high performance liquid chromatography (HPLC)

The HPLC analyses were carried out on a Hitachi Chromaster (Pump 5110, Auto sampler with thermostat 5210, Diode Array detector-DAD 5430 and Column oven 5310). The chromatographic conditions were as follows: Column: Symmetry C18 (250 mm x 4.6 mm; 5 μm i.d., Waters, USA), column temperature: 30 °C, sample volume 20 μL, UV detection at

280 nm, mobile phase: phosphate buffer (pH 3.0) and methanol in ratio of 23:77 v/v, flow rate: 1.2 mL/min. The identification and the quantification of capsaicin and dihydrocapsaicin was performed by using external standard method. The total capsaicinoid quantity in extract expressed in mg/g extract or % in extract was calculated as sum of contents for capsaicin and dihydrocapsaicin. The HPLC chromatogram of the extract of red hot pepper sample is presented in Fig. 4.10.

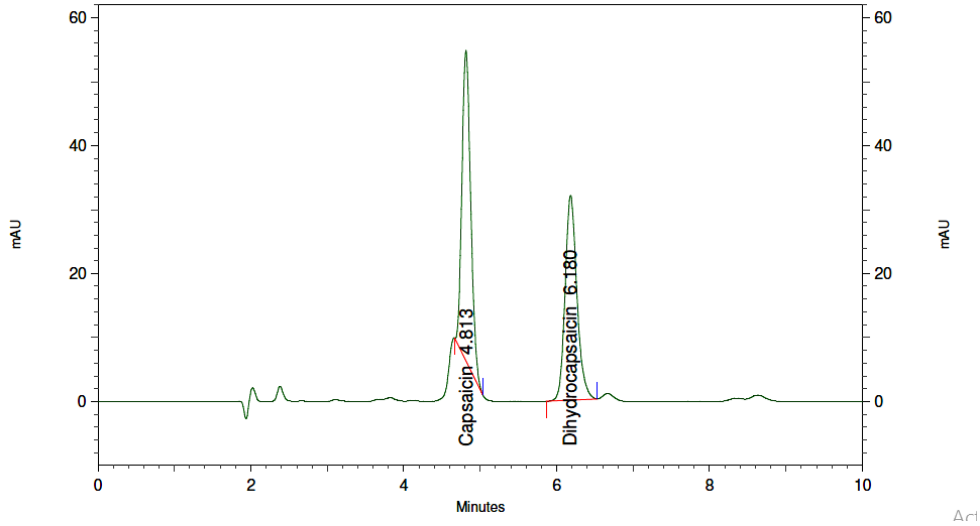


Fig. 4.10. HPLC chromatogram of the extract of red hot pericarp.

4.4.4.3. Determination of capsaicin by ultraviolet-visible spectrometry (UV-VIS)

The capsaicin was quantified by measuring the absorbance at 282 nm of extract of red hot pepper dissolved in ethanol. The equation of calibration curve prepared with standard capsaicin solutions in ethanol was used, $A = 0.0144\ \gamma - 0.0091$ (R^2=0.9995), where γ is μg capsaicin in mL extract and *A is* absorbance (Pino et al., 2007). UV–Vis spectra of standard capsaicin and extract of red hot pepper are presented in Fig. 4.11.

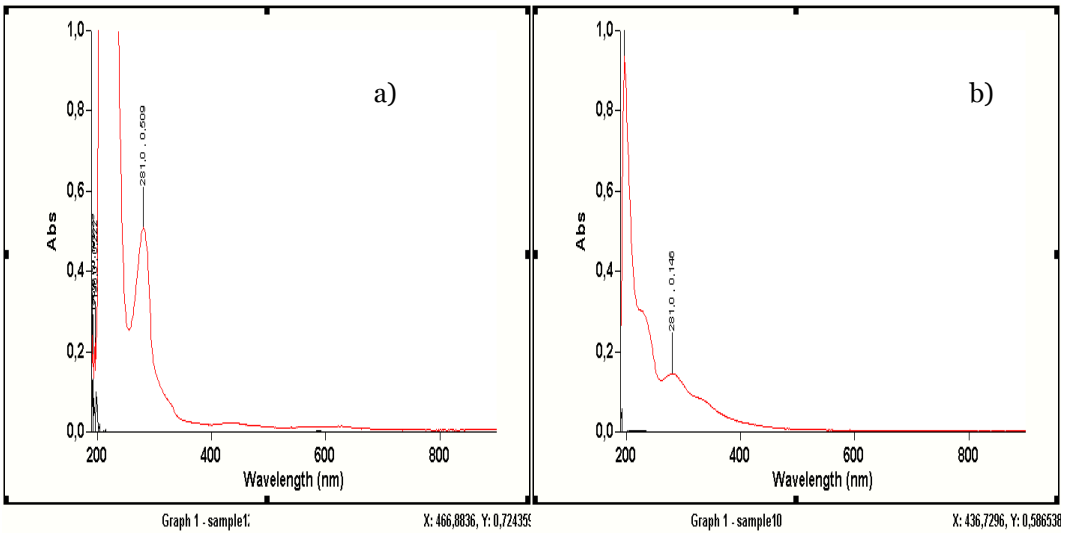


Fig. 4.11. UV–Vis spectrum of standard capsaicin (a) and extract of red hot pepper (b).

4.4.5. Determination of carotenoids

4.4.5.1. Determination of total carotenoids

a) The plant material extract (0.5 ml) was dissolved in 10 ml of acetone for the determination of the carotenoid content. The absorbance was measured at wavelength of 460 nm on a Varian Cary Scan 50 spectrophotometer (Switzerland) in 1cm quartz cells, at 25 °C. The carotenoid content was calculated using the extinction coefficient in acetone of the major carotenoid capsanthin ($^{1\%}E_{460nm}$= 2300) in red pepper (Hornero-Méndez et al., 2000).

b) 50 mg extract was weight into 100 mL volumetric flash approximately and 25 mL of acetone was added. The flask was briefly vortexed and filled up to volume with acetone. If expected carotenoids concentration in oleoresin is higher than 7%, the extract was diluted with acetone (1:10 v/v). The max absorbance against an acetone blank was read on spectrophotometer.

4.4.5.2. Determination of *β*–carotene

0.1 g extract was dissolved in a 25 mL solvent mixture of dichloromethane and methanol (25:75 v/v). 6 mL 0,107 M NaOH dissolved in methanol freshly prepared was added to 25 mL extract solution under internet atmosphere. The solution was kept -4° C for 16 h for complete hydrolysis of carotenoids esters. The saponified pigments extract solution was filtered through membrane filter with 0.45 µm pore size prior the HPLC.

The HPLC analyses were carried out on a Hitachi Chromaster (Pump 5110, Auto sampler with thermostat 5210, Diode Array detector-DAD 5430 and Column oven 5310). The pigments were separated on Waters C18 normal phase column (250 x 4.6 mm, 5 µm) at room

temperature. Aliquots of 20 µL were used for HPLC analyses. The mobile phase was consisted of eluent A (dicholomethain: acetone: acetonitrile: water = 5:85:5.5:4.5 v/v) and eluent B (dicholomethain: acetone: acetonitrile: water = 25:28:42.5:4.5 v/v). The separation of carotenoids was achieved by following the gradient procedure: 0% B for 8 min; linear gradient from 0 to 100% of 6 within 6 min; 100% B for 40 min at the flow rate 1 ml/min. Peaks were measured at wavelength of 480 nm. The identification and the quantification of β-catotene was performed by using external standard method.

4.4.6. Determination of fatty acids

Fatty Acid Methyl Esters (FAME) Preparation. The aliquots (0.1 g) of oleoresin was measured in screw tube. 1 mL hexane, 2 mL BCL3-methanol (12%w/w) and 0.5 mL 2, 2–dimethoxypropane (DTP) were added in the extract. The mixture was heated on 60 °C for 10 minutes. After the cooling, 1 mL water (MiliQ) and 1 mL hexane were added. The screw tube was shacked (Vortex) in order to get the esters in non-polar solvent. The layer was settled, the upper layer – organic part was carefully transferred into clean vial. The organic layer was dried by adding anhydrous sodium sulfate into clean 5 mL vial and then shaking. The solution (2 mL) containing methyl esters of fatty acid filled to 10 ml volume flask with hexane. Chromatographic conditions. The gas chromatograph Shimadzu GC 2010-Plus (Japan) equipped with an automatic liquid sampler (AOC-20I+S) and a flame ionization detector (FID) was used with a ZB-FFAP column (30 m x 0.25 mm ID x 0.25 m, Phenomenex, USA). The carrier gas was He (3.0 mL/min flow rate) and the split ratio was 1:50. The injection temperature was maintained at 250 °C and the FID at 260 °C. The oven temperature was set at 180°C (3 min) increasing by 2 °C/min. The final oven temperature was maintained at 240 °C (25 min). The aliquots (1.0 µl) of the derivatized extracts were injected into the column. The identification of the individual fatty acid methyl esters was achieved by comparison with reference standards (F.A.M.E. Mix RM-6, Sigma-Aldrich Co., USA).

4.4.7. Fourier transform infrared ray (FTIR) spectroscopy

Attenuated total reflectance (ATR) spectra were collected by Varian 660 FT-IR spectrometer (CA, USA) with MIRAcle ZnSe ATR module (PIKE technologies) with low pressure micrometer clamp. The FTIR spectra were acquired in the range of 4000–550 cm^{-1} region at a resolution of 4 cm^{-1} by accumulation of 32 scans.

4.4.8. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA/DTA)

DSC analysis was performed with NETZSCH DSC 204 F1 instrument, in temperature range from 0 to 250 °C with heating rate of 10 °C/min. The measurements were carried out under dynamic nitrogen atmosphere (30 mL/min) in pierced aluminum pans. TGA/DTA based on EN ISO 11358 standard (2014) was performed by using *Perkin Elmer* DIAMOND system (Japan) in the nitrogen atmosphere, temperature range from 25 to 1000 °C, with a heating rate of 20 °C/min.

4.4.9. Edible film characterization

4.4.9.1. Thickness measurement

Thickness of the films was measured using a hand-held micrometer. Eight thickness measurements were taken at random positions on each film, and the mean value was calculated.

4.4.9.2. Water vapor permeability (WVP)

The water vapor permeability was measured gravimetrically according to the ASTM E96/E96M-12 (1993) protocol. The films were sealed with silicone to the top of a glass petri dish with a diameter of 5 cm. The water vapor permeability was measured using films conditioned previously at a relative humidity of 54%. A fan was used to promote the circulation of air inside the desiccator, in order to minimize the mass transfer resistance of the air boundary layer above the membrane. The room temperature and the relative humidity outside the petri dish were measured over time using a thermohygrometer. The water vapor molar flux (N) was determined by weighing the petri dish in regular time intervals for 24 h. The water vapor permeability was calculated as is given in equation (4.1).

$$WVP = \frac{N \cdot \delta}{\Delta Pw} \qquad (4.1)$$

where δ is the film thickness (m) and ΔP_w is the water vapor difference between both sides of the film.

4.4.9.3. Mechanical properties

The mechanical properties were studied using a TA–XTplus (Stable Micro Systems, England). For the puncture test, five sample squares (20 x 20 mm) of the films were cut out from the conditioned films. The puncture was carried out with probe (P/2) with 2 mm diameter, while the film squares were fixed down on perforated heavy duty platform. The initial distance was set to be 5 mm. The films were punctured in the center with cross-head speed of 1 mm/s, and the force and distance necessary to puncture the films were recorded. Puncture tension expressed in MPa was calculated by dividing the maximum force by the cross-sectional area of the probe, while the deformation was calculated as a ratio of the deformation at the point of sample rupture to the set initial distance as a percentage. Texture data were processed using the following equations (4.2-4.4)

$$L = \sqrt{Li^2 + d^2} \tag{4.2}$$

$$\varepsilon(\%) = \left(\frac{L-Li}{Li}\right) . 100 \tag{4.3}$$

$$\sigma = \frac{F_{max}\,(N)}{1 . 10^{-4}(m^2)} \tag{4.4}$$

where: L – distance of elongation at puncture (mm), L – initial distance (mm), d – probe diameter (mm), ε – sample deformation (%), σ – puncture tension (MPa), F_{max} – puncture force (N).

4.4.9.4. Optical properties

Light transmission and film transparency. The ultraviolet (UV) and visible light barrier properties were measured on dried films at selected wavelengths (in the 200–800 nm range), using an UV-VIS Spectrophotometer type Cary 50 Scan (Switzerland). The film samples were cut into strips (9 × 45 mm) and were attached to one side of a quartz cuvette, while the quartz cuvette was used as control. The relative transparency of the film was measured at 600 nm, and transparency was calculated according to Eq. 4.5

$$\text{Transparency} = A_{600}/\delta \tag{4.5}$$

where: A_{600} – absorbance at 600 nm, δ – the film thickness (mm). At least five strips of each film type were analyzed.

Colour. The film colour was evaluated using Dr. Lange spectra colorimeter (UK) presented in Fig. 4.12. A CIELab colour scale was used to measure the degree of L* (lightness–darkness), a* (red–green), b* (yellow–blue) of the films, under D65 (daylight) and 10° observer angle. Film specimens were measured on the surface of the white standard plate, with colour coordinates $L^*_{standard}$= 95.93, $a^*_{standard}$= −0.19 and $b^*_{standard}$= 3.12.

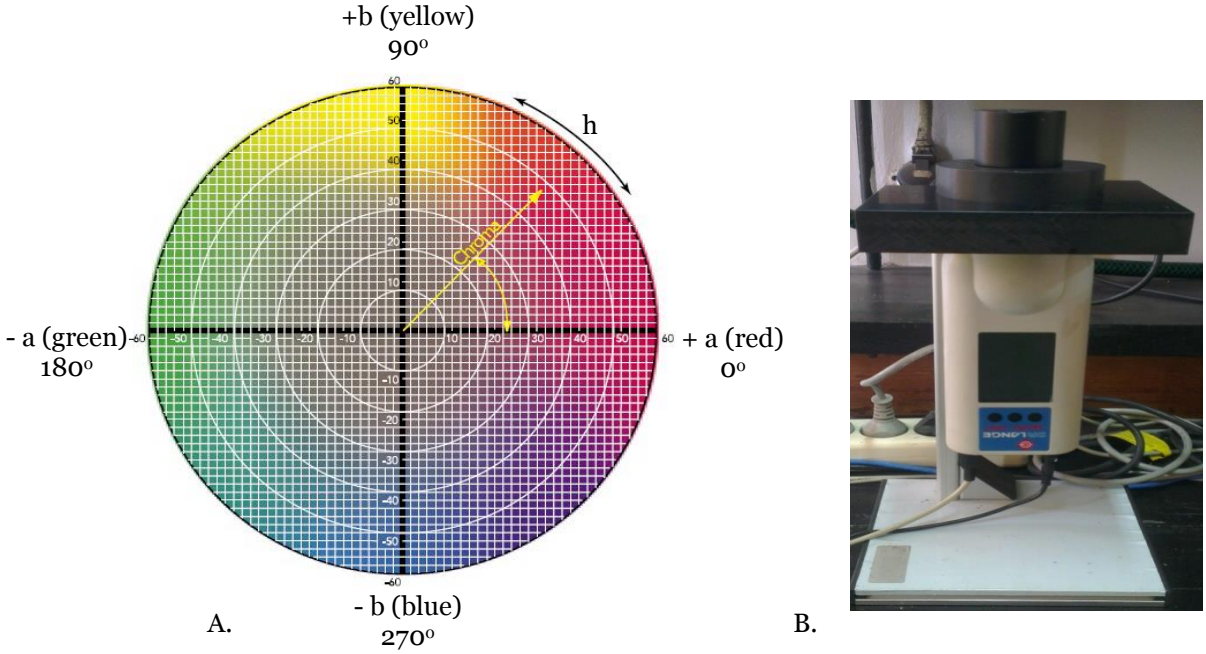


Fig. 4.12. CIE L*a*b color scheme (A) and Dr Lange colorimeter (B).

Hue angle (H) is derived from the coordinates a* and b* and determined as arctang b*/a*. Chroma (C) as a measure of intensity or saturation is calculated as $(a^* + b^*)^{½}$.

The colour of each film was expressed as the total difference in colour (ΔE), and whiteness index (*WI*) were calculated according to equation 4.6 and 4.7.

$$\Delta E = [(L^*_{film} - L^*_{standard})^2 + (a^*_{film} - a^*_{standard})^2 + (b^*_{film} - b^*_{standard})^2]^{1/2} \quad (4.6)$$

$$WI = 100 - [(100 - L^*_{film})^2 + (a^*_{film})^2 + (b^*_{film})^2]^{1/2} \quad (4.7)$$

4.4.9.5. Antimicrobial activity

The antimicrobial activity of edible film with capsaicin was studied using agar diffusion method following the procedure described by Pranoto et al. (2005). The microorganisms used in this experiment were isolated from two commercial soft white

cheeses, one produced from sheep's and the other from cow's milk. The cheeses were left open (out of package) at refrigeration temperatures (4-7°C) for two weeks during which microbial growth was noticed on the surface. The infected material was diluted several times in 0.1 % peptone water and seeded on *Sabouraud*–dextrose and *Nutrient* agar (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) using the Koch method. The grown colonies were then inspected for their cultural and microbiological characteristics and isolated as pure cultures.

Five edible films were prepared using different amount of seed extract of red hot pepper into final concentration in the films of: 12, 20, 30, 50, 70 mg/mL. The films were then cut into 5 mm diameter disks and placed on *Sabouraud*–dextrose agar which had previously been seeded with 0.1 mL cell suspension containing 10^5 CFU mL^{-1} of each target microorganism. Film disks without incorporated capsaicin seed extracts were used as negative controls. The tests ware performed in duplicate, in two separate experimental runs. The sensitivity of the microorganisms to the seed extract was rated based on the diameter of the generated inhibition zone.

4.4.10. Nanoemulsion characterization

Droplet size and *droplet size distribution*, *polydispersive index* as well as *zeta-potential* were determined by DLS using a Zetasizer Nano ZS (Malvern Instruments Ltd, United Kingdom) device. The stability of emulsion was measured at 24h after preparation, followed at temperature of 4 and 25°C and after 10 days of preparation on 25°C. Droplet size and droplet size distribution as well as zeta-potential were determined by using a Zetasizer Nano ZS (Malvern Instruments Ltd, United Kingdom) device (Pecora R., 2000). The measurements were conducted at 25 °C. Each sample was measured on Zetasizer Software Version 7.11, in three successive runs and in each run the sample was scanned ten to 100 times for the potential, allowing one to produce accurate size determination results. At high energy method the drop disttibution was measured by Mastersizer 2000 apparatus (Fritarom, Etol, doo, Slovenia). the samples were dissolved with water (1:50 w/w) and measured droplet size and, distribution, as well as zeta-potential using a Zetasizer Nano ZS (Malvern Instruments Ltd, United Kingdom) device (Pecora R., 2000).

Morphological appearance of nanoemulsions was observed using environmental scanning electron microscopy (ESEM) type Quanta FEI 200 3D (USA). ESEM, microscope Quanta FEI 200 3D in environmental mode, provides a relatively new technology for imaging materials with-out specimen preparation and conductive coating. The cells were mounted on aluminium stubs and direct observed in their natives tate. The pressure chamber was around 83 Pa and an accelerating voltage for imaging was 15 kV.

4.4.11. Biopesticide characterization

A *laboratory trial* was performed on the infested tobacco leaves with aphid degree of 4 on *Petri* dishes. According to the scale of the Manual on integrated protection it means that on average each leaf were 51 to 100 aphids in different stages of the life cycle (from 204 to 400 aphids). The extracts and imidacloprid were sprayed on tobacco leaves with a mini pressure spryer (Fig. 4.13). After 24 hours of imidacloprid and extracts application, the number of aphids on leaf was counted The efficacy of the applied extracts and imidacloprid was estimated according to *Abbott*'s formula, Eq. 4.8 (WHO, 2009).

a) tobacco leaves in Petri dish b) Spraying of biopesticide

Fig. 4.13. Laboratorory trial for control of aphid growt (*Myzus persicae* Sulz) on tobacco leaves.

$$\textbf{Efficiency}\ (\%) = \left(1 - \frac{N_T}{N_K}\right) \cdot 100 \qquad (4.8)$$

where: N_T – number of aphids after treatment of 4 leaves with pesticide, N_K – total number of aphids on 4 leaves.

The *experimental trial* was performed on whole tobacco plants in greenhouse, 5 tobacco plants were treated with extracts. The upper 4 leaves were examined for the aphid population mortality (Fig. 4.14). *Schneider-Orelli*'s formula, Eq. 4.9 (WHO, 2009) was used in the aphid mortality determination in greenhouse experiment after 24 and 48 hours treatment with extracts and imidacloprid insecticide.

Fig. 4.14. Greenhouse trial of efficacy of pepper extracts.

$$\textbf{Mortality}\ (\%) = \frac{C_T - C_k}{100 - C_k} \cdot 100 \qquad (4.9)$$

where: C_T – mortality of aphids of treated plants (%), C_T – mortality of aphids of control, untreated plants (%),

4.4.12. Statistical analysis

STATISTICA 8 software (StatSoft, Inc., USA) was used in the statistical analysis of the obtained results. The data were analyzed with one-way ANOVA followed by multiple comparisons with Tukey's honest significant difference (HSD) test at the 5% significance level ($p < 0.05$).

5. RESULTS AND DISCUSSION

5.1. PLANT MATERIAL CHARACTERISTICS

5.1.1. Red hot pepper fruit composition

A. PERICARP, SEED and PLACENTA. The chemical characteristics of the separated pericarp, seed and placenta from the dried fruits of red hot pepper with the particle size of 0.25 mm are presented in Table 5.1. The content of proteins expressed in relation to the corresponding DM was higher in placenta (25.19%). The determined cellulose content in the pericarp and seed was 26.37% and 22.66%, respectively. The content of reductive sugars in the pericarp and placenta was almost six times higher than in the seed, where 2.96% of the reductive sugars were found. Zacharian and Gobinath (2008) reported a protein content of 14-16%, ~8% ash, 0.1% calcium and 0.90% iron in the pericarp of hot *Capsicum*. An ash content of 4.22 g/100 dry mass (DM) in the fruits of hot pepper was determined by Ozgur *et al.* (2011). The literature data for the chemical composition of dried red hot peppers show the following contents: protein 109 g/ kg, carbohydrate 462 g/ kg, calcium 1230 g/ kg, phosphorus 1400 g/ kg and iron 1000 g kg^{-1} (Ku and Choi, 1990).

Table 5.1. Chemical composition in different parts of red hot pepper (0.25 mm particle size).

Characteristic	Sample		
	Pericarp	Seed	Placenta
Dry matter (%)[1]	$95.60^{a} \pm 0.82$	$95.50^{a} \pm 0.94$	$94.50^{a} \pm 0.72$
Proteins (%)*[1]	$14.13^{a} \pm 0.36$	$20.88^{b} \pm 0.75$	$25.19^{c} \pm 0.35$
Ash (%)*[1]	$16.32^{a} \pm 0.29$	$3.76^{b} \pm 0.41$	$17.99^{c} \pm 0.50$
Sand (%)*[1]	$0.52^{a} \pm 0.03$	$2.61^{b} \pm 0.19$	$0.40^{a} \pm 0.05$
Cellulose (%)*[1]	$26.37^{a} \pm 0.57$	$22.66^{b} \pm 0.69$	$15.05^{c} \pm 0.46$
Reductive sugars (%)*[1]	$19.73^{a} \pm 0.82$	$2.96^{b} \pm 0.38$	$20.42^{a} \pm 0.73$
Carotenoids (%)*[1]	$0.42^{a} \pm 0.04$	/	$0.37^{a} \pm 0.05$

*Calculated according to the corresponding dry matter (DM). [1]Data are expressed as mean ± standard deviation (n = 3). [a,b,c] Values with different superscripts within a row are significantly different (Tukey test, $p < 0.05$).

Content of carotenoids in red hot pepper fruits. In the pericarp and placenta of dried red hot pepper, the content of carotenoids was 0.42 and 0.37% DM, respectively (Table 5.1). In mature pepper fruits, the total carotenoid contents in the range between 0.69 to 13.85 mg/g DM were determined by Troconis-Torres et al. (2012). The Habanero chili has a lower content of carotenoids, 14 mg/g DM, than Chiltepin chili fruits, 36 mg/g DM (Rodríguez-Maturino et al., 2012). Ozgur *et al.* (2011) published a higher capsanthin content in sweet red pepper (2282.45 mg capsanthin/kg) in comparison to the capsanthin value of 996.38 mg/kg determined in a hot variety of *Capsicum annuum* L. from Lee *et al.* (2004). The capsanthin quantity in hot peppers varies from 17.55 to 109.5 mg/kg (Orak and Demirci, 2005).

Macro and microelement content in hot pepper fruits. In Table 5.2. is given the summarized content of macro and microelements determined in different parts of the analyzed red hot pepper fruits. The determined quantity of potassium in the pericarp and placenta was around 5230 mg/ kg DM. In terms of the contents of Cu, Zn, Mn and P, the highest amounts were found in the seed. In comparison to the pericarp and seed, the placenta was characterized by the highest quantities of Fe (916.7 mg/kg DM), Mg and Ca (4572.9 and 1767.8 mg/ kg DM, respectively).

Table 5.2. Macro and microelements (mg/kg DM)*[1] in different parts of red hot pepper.

Element	Sample		
	Pericarp	Seed	Placenta
K	$5232.13^{a} \pm 2.55$	$5209.93^{b} \pm 2.77$	$5230.50^{a} \pm 2.08$
Cu	$13.42^{a} \pm 0.53$	$20.68^{b} \pm 0.77$	$19.22^{b} \pm 0.62$
Zn	$46.42^{a} \pm 1.00$	$70.32^{b} \pm 0.89$	$61.73^{c} \pm 1.08$
Mn	$7.94^{a} \pm 0.73$	$38.91^{b} \pm 1.03$	$15.16^{c} \pm 0.66$
Fe	$446.24^{a} \pm 1.56$	$195.86^{b} \pm 2.03$	$916.72^{c} \pm 1.60$
Mg	$3309.61^{a} \pm 2.67$	$4176.67^{b} \pm 2.57$	$4572.95^{c} \pm 2.59$
Ca	$944.51^{a} \pm 1.92$	$483.13^{b} \pm 1.17$	$1767.87^{c} \pm 2.06$
P	$3.15^{a} \pm 0.08$	$3.21^{a} \pm 0.10$	$2.60^{a} \pm 0.70$

*Calculated according to the corresponding dry matter (DM). [1]Data are expressed as mean ± standard deviation (n = 3). [a,b,c] Values with different superscripts within a row are significantly different (Tukey test, $p < 0.05$).

Content of capsaicinoids in red hot pepper fruits. The capsaicinoid contents determined in the pericarp, seed and placenta differed significantly (Table 5.3). The highest content of capsaicin was found in the placenta, as well as dihydrocapsaicin, 10.48 and 6.43 mg/g DM, respectively. The highest ratio of 3.71 estimated from the quantity of capsaicin and dihydrocapsaicin was calculated in the pericarp. The determined pungency level in placenta of 272 211 SHU was almost five times and two times higher than the pungency level in the seed and pericarp, respectively.

Table 5.3. Content of capsaicinoids in different parts of red hot pepper.

Sample	**Capsaicin** (mg/g[1])*[1]	**Dihydrocapsaicin** (mg/g)*[1]	**Capsaicin and dihydrocapsaicin ratio**	**Quantity of total capsaicinoids** (%)	**Scoville heat values** (SHU)
Pericarp	$5.38^{a} \pm 0.44$	$1.45^{a} \pm 0.20$	3.71^{a}	25.06^{a}	$109\,923^{a}$
Seed	$2.36^{b} \pm 0.06$	$1.15^{a} \pm 0.01$	2.05^{b}	12.87^{b}	$56\,431^{b}$
Placenta	$10.48^{c} \pm 0.22$	$6.43^{b} \pm 0.15$	1.63^{c}	62.07^{c}	$272\,211^{c}$

*Calculated according to the corresponding dry matter (DM). [1]Data are expressed as mean ± standard deviation (n = 3). [a,b,c] Values with different superscripts within a row are significantly different (Tukey test, $p < 0.05$).

As reported in the literature, the content of capsaicinoids is affected by the variety of the hot pepper, as well as by environmental conditions and maturity. Variations in the total capsaicinoid content have been reported by many authors in relation to the influence on the red hot pepper variety. The capsaicin content in ripe fruits of Indian hot pepper cultivars

varies from 37.6 to 497.0 mg in 100 g, with the corresponding level of pungency from 15 000 to 300 000 SHU (Gibbs and Garro, 2004). The quantity of total capsaicinoids was 40.07 mg in 100 g with 750 000 SHU in red hot peppers as determined by Lee et al. (2004). In the fruits of *Capsicum* varieties cultivated in India, Sanatombi and Sharma (2008) established the amount of capsaicin from 0.14 to 0.17% in *Capsicum annuum*, 0.65–0.88% in *Capsicum frutescens* and 0.79–2.06% in *Capsicum chinense*. According to the high content of capsaicinoids found, *Capsicum chinense* was classified in a high pungency level group (Antonious et al., 2009; Othman et al., 2011). Higher concentration of capsaicin (9.177 ± 0.268 mg g^{-1}) and pungency level (146 823 SHU) was established in "Nsukka" *Capsicum chinense* yellow pepper compared to "Zaria tatashe" *Capsicum annuum* (Nwokem et al., 2010). In the fresh fruits of the scotch bonnet variety of hot pepper, Gahungu *et al.* (2011) found capsaicin and dihydrocapsaicin quantities of 47.63 mg/g and 23.10 mg/g, respectively. The total capsaicinoid content ranged from 525.7 μg/g DM for Chipotle to 3330.9 μg/g DM for Serrano hot pepper (Alvarez-Parrilla et al., 2011). López *et al.* (2012) found that *Capsicum chacoense* Hunz. contains a higher amount of capsaicin (13.9 mg in 100 g DM) than *Capsicum baccatum* L. (12.6 mg in 100 g DM) and *Capsicum annum* L. (10.1 mg in 100 g DM). The total contents of the capsaicinoids were found in the range between 1758.2 and 7068.9 μg/g DM, which corresponds to Scoville heat values in the range between 26 400 and 106 000 SHU (Juangsamoot et al., 2012). In mature fresh pepper, Perucka and Materska determined that the capsaicin and dihydrocapsaicin contents ranged between 0.28 and 0.59 mg/g DM and 0.12 and 0.34 mg/g DM, respectively (Perucka and Materska, 2001). Moreover, Kraikruan *et al.* (2008) informed that capsaicin and dihydrocapsaicin contents were the highest in the first harvest in all cultivars and then they decreased in the subsequent harvests. The highest capsaicin content in fruits was found in cultivars grown at a high temperature and in nutrient-rich soils (Sung et al., 2005; Rahman, and Inden, 2012). Johnson and Decoteau (1996) suggested that the levels of capsaicin and dihydrocapsaicin increase proportionally as the amount of nitrogen fertilizers increases. The highest content of capsaicin (227 mg/100 g of fresh weight) in placenta was found in the "Takanotsume" cultivar (Supalkova et al., 2007). According to the distribution of capsaicinoids in different parts of the fruits, Canto-Flick et al. (2008) determined the total capsaicinoid content in the whole fruit, placenta, and pericarp of 18 accessions of Habanero pepper. The placenta of Tunisian red hot pepper varieties has the highest content of capsaicinoids (2.32 to 0.065 mg/g DM) compared to the content of the pericarp and seed, where the determined quantity of capsaicinoids was very low, approximately 0.5% in pericarp and 0.1% in seed (Mansour-Gueddes et al., 2012). According to the total content of capsaicinoids and determined Scoville heat values, the following classification of peppers was proposed (Govindarajan, 1986; Weiss, 2002): sweet non-pungent varieties (0.1 to 0.2%, 0–5 000 SHU), moderately pungent (0.2–

0.4%, 5 000–20 000 SHU), pungent (0.4 to 0.6%, 20 000–70 000 SHU) and very pungent (to 1.4%, 70 000–300 000 SHU).

B. STALK. The summative chemical composition of the separated stalk from dried fruits of red hot pepper are presented in Table 5.4. Generally, the influence of the particle size on the difference in the chemical composition is insignificant. The content of proteins expressed in relation to the corresponding DM varied from 17.73 to 17.78%. The fat content increases by decreasing particle size. The highest value for fat (3.70% DM) was determined in stalk with 0.25 mm particle size. The determined quantity of ash and cellulose was around 10 and 26% DM, respectively. The content of reductive sugars decreased from 6.31 to 6.16% by increasing the particle size from 0.25 to 1.0 mm. The energetic contribution for red hot pepper stalk with particle size of 0.25, 0.5 and 1.0 mm expressed in kcal/100 g DM was 195.80, 188.47 and 180.63, respectively.

Table. 5.4. Chemical composition and surface colour data of the red hot pepper stalk.

with different particle size. **Characteristic**		**Particle size** (mm)		
		0.25	0.5	1.0
Moisture content (%)[1]		$7.84^{a} \pm 0.06$	$8.02^{a} \pm 0.08$	$8.01^{a} \pm 0.11$
Proteins (%)[1,2]		$17.77^{a} \pm 0.03$	$17.73^{a} \pm 0.05$	$17.78^{a} \pm 0.09$
Fats (%)[1,2]		$3.70^{a} \pm 0.12$	$2.98^{b} \pm 0.10$	$1.97^{c} \pm 0.06$
Ash (%)[1,2]		$9.75^{a} \pm 0.05$	$9.68^{b} \pm 0.04$	$9.79^{b} \pm 0.02$
Cellulose (%)[1,2]		$25.91^{a} \pm 0.08$	$25.71^{ab} \pm 0.03$	$25.87^{b} \pm 0.02$
Reductive sugars (%)[1,2]		$6.31^{a} \pm 0.02$	$6.19^{b} \pm 0.02$	$6.16^{b} \pm 0.05$
Cu (mg/kg)[1,2]		$9.16^{a} \pm 0.06$	$9.06^{a} \pm 0.07$	$8.25^{a} \pm 0.08$
Zn (mg/kg)[1,2]		$14.05^{a} \pm 0.19$	$13.77^{a} \pm 0.21$	$10.71^{b} \pm 0.41$
Mn (mg/kg)[1,2]		$90.90^{a} \pm 2.57$	$71.86^{b} \pm 1.60$	$71.01^{b} \pm 1.67$
Fe (mg/kg)[1,2]		$175.44^{a} \pm 2.58$	$163.55^{b} \pm 2.35$	$132.47^{c} \pm 1.42$
Extract yield (g/kg)[1,2]	*E*	$236.77^{a} \pm 0.82$	$112.31^{b} \pm 0.55$	$54.77^{c} \pm 0.76$
	H	$95.89^{a} \pm 0.30$	$63.69^{b} \pm 0.46$	$39.64^{c} \pm 0.54$
Capsaicin (mg kg)[1,2]	*E*	$28.75^{a} \pm 0.68$	$23.00^{b} \pm 0.66$	$22.14^{b} \pm 0.46$
	H	$9.28^{a} \pm 0.46$	$6.16^{b} \pm 0.44$	$2.22^{b} \pm 0.116$
Total carotenoids (mg/kg)[1,2]	*E*	$228.15^{a} \pm 3.61$	$272.34^{a} \pm 1.36$	$338.38^{b} \pm 5.07$
	H	$344.19^{a} \pm 22.59$	$443.32^{b} \pm 13.10$	$721.10^{c} \pm 15.90$

[1]Data are expressed as mean ± standard deviation (n = 3); [2]Calculated according to the corresponding dry matter (DM); *E*–Ethanol; *H*–*n*-Hexane; [a,b,c] Values with different superscripts within a row are significantly different (Tukey test, $p < 0.05$).

Krstic *et al.* (2013) reported higher content of cellulose in the stalk of hot pepper varieties (20.29% DM) than in sweet varieties (17.13% DM). In comparison to the pericarp and the seed from the same variety of red hot pepper Simonovska et al., 2014, the stalk is richer with proteins (17.77%) and ash (3.76%). The cellulose content in stalk samples is higher than the cellulose value in the seed (22.66%) and placenta (15.05%). The stalk has higher content of reductive sugars as the seed (2.96%). The highest contents of cuprum (Cu), zinc (Zn), manganese (Mn) and iron (Fe) were found in the stalk with particle size of 0.25 mm. In comparison to the pericarp and seed, the placenta was characterized by the highest quantities of Fe (916.7 mg kg^{-1} DM), Mg and Ca (4572.9 and 1767.8 mg kg^{-1} DM, respectively).

The stalk has a lower content of Cu, Zn and Fe and higher content of Mn than pericarp, placenta and seed.

Extractives and content of capsaicin and colour compounds. In Table 5.4 are presented the results for quantity of extractives, and content of capsaicin and total carotenoids in red hot pepper stalk. Concerning the quantity of extractives obtained by ethanol and *n*-hexane, the major proportion corresponds to polar compounds was solubilized by ethanol. The highest extract quantity (236.77 g/kg DM) was obtained from stalk with 0.25 mm particle size when ethanol was applied as solvent. Ethanol was more effective in the extraction of capsaicin, while *n*-hexane in extraction of total carotenoids. No data were found in literature related to the quantity of extractives and carotenoid content in red hot pepper stalk. The published data for the pericarp of the red hot pepper cultivars indicate that methanol and ethanol are more appropriate in extraction of bigger quantity of extract richer with capsaicinoids, while *n*-hexane and acetone are suitable for pigments extraction (Rafajlovska et al., 2011). Chen et al. (2014) reported that stalk extract obtained with methanol is richer in capsaicin than placenta and seed extract.

Thermogravimetric (TG/DTG), Differential scanning calorimetry (DSC) and FTIR data. Fig. 5.1 shows the thermal stability curve (TGA) and the derivative of the TGA weight loss curve, or the rate of weight loss (DTG) for the red hot pepper stalk with particle size of 0.25, 0.5 and 1 mm. Similar shaped TGA curves obtained for all samples showed a four-stage weight loss below 600 °C. The initial sharp slope in the first region, starting from room temperature up to 100 °C corresponds to the water loss (drying). The moisture content was in the range from 2 % for 1 mm stalk to 3% for 0.5 mm stalk which is comparable with the data of Ali et al. (2014). In the second rather narrow region from 100 to 170 °C, the stalk samples experienced the first weight loss (about 6% of the sample) decomposed into volatiles because of the thermal decomposition of low molecular weight components. Generally, the cellulose degradation occurs in the range of 240 to 350 °C (Poletto et al., 2014). In the third region in the temperature range of 210 to 230 °C, the cellulose in the stalk starts to decompose slowly with 16% of the weight loss. At the temperature around 304°C hemicellulose continues to decompose with weight loss of 30-33%. In the temperature range from 392 to 433 °C the lignin components in the stalk samples decompose with around 50% weight loss. Corresponding DTG curves (Fig. 5.1) show a series of maxima related to vaporization of water or to decomposition of stalk compounds. The stalk sample with smaller particle size (0.25 mm) showed higher thermal stability with higher initial and final degradation temperature, as well as higher residual weight.

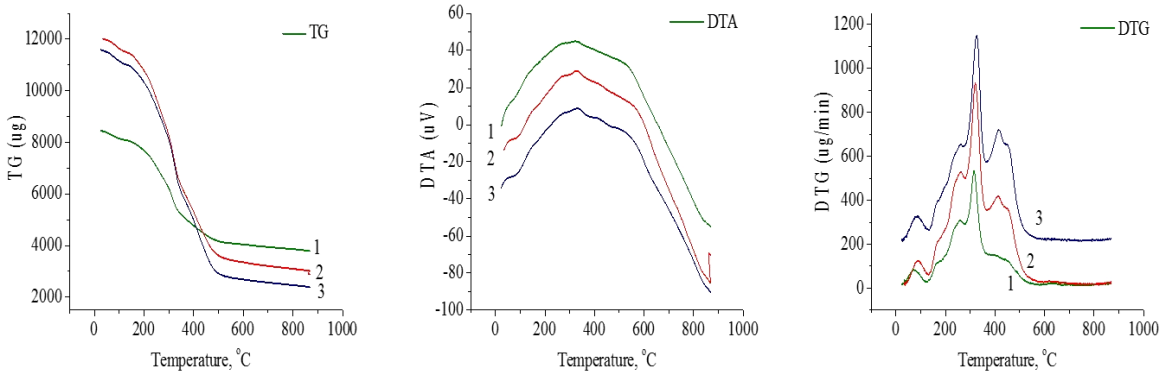


Fig. 5.1. TGA/DTA curves of red hot pepper stalk with particle size of 0.25 mm (1), 0.5 mm (2) and 1.0 mm (3).

DSC curves of the red hot pepper stalk samples with particle size of 0.25, 0.5 and 1 mm are presented in Fig. 5.2. The characteristic glass transition temperature (*Tg*) was determined in the range of 49 to 56°C. It increases by increasing the particle size (*Tg*= 56°C for stalk 1 mm; *Tg* = 52 °C for stalk 0.5mm; *Tg* = 49 °C for stalk 0.25mm). It is evident that *Tg* slightly decrease by decreasing the stalk particle size of red hot pepper stalk (Chen et al., 2012).

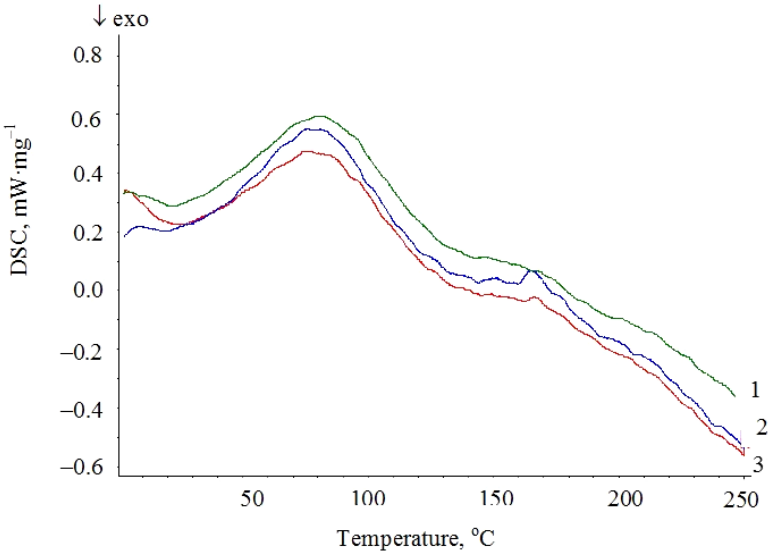


Fig. 5.2. DSC curves of red hot pepper stalk with particle size of 0.25 mm (1), 0.5 mm (2) and 1.0 mm (3).

The FTIR spectra of different particle size fractions of red hot pepper stalk are presented in Fig. 5.3. Spectra display a number of adsorption peaks indicating the complex nature of this material. The broad peak at around 3300-3400 cm^{-1} is indicative of OH vibration modes. The two sharp peaks at 2920 cm^{-1} and 2880 cm^{-1} correspond to the asymmetric and symmetric vibration, respectively, C-H in the olephinic chains, and the peak

at 1743 cm^{-1} is attributed to the carbonyl C-O in ester groups. The presence of lignin was confirmed by the typical lignin bands at 1323 cm^{-1}, 1270 $cm^{-1}$and 1600 cm^{-1}, being the first two bands attributed to skeletal vibrations of aromatic rings with CO stretching, respectively, and the last one to aromatic skeletal vibrations (Wang et al., 2012). The band at 1450 cm^{-1} associated to deformation vibration of C-H in aromatic ring of lignin moieties is less intense. The presence of the typical peaks of polysaccharides appear at 1075, 1118 and 996 cm^{-1}. The comparison among the different FTIR spectrum of different particle size fractions of red hot pepper stalk show that the above mentioned peaks are present in all fractions. Slight differences in the 2870 cm^{-1} band intensity can be observed in the finest particle size fraction compared to the other two ones. For the finest fraction (0.25 mm), the most relevant differences are observed in the bands attributed to polysaccharides (1075, 1118 and 996 cm^{-1}) and to lignin (1525 and 1450 cm^{-1}) that show lower peak intensities compared to the other two fractions.

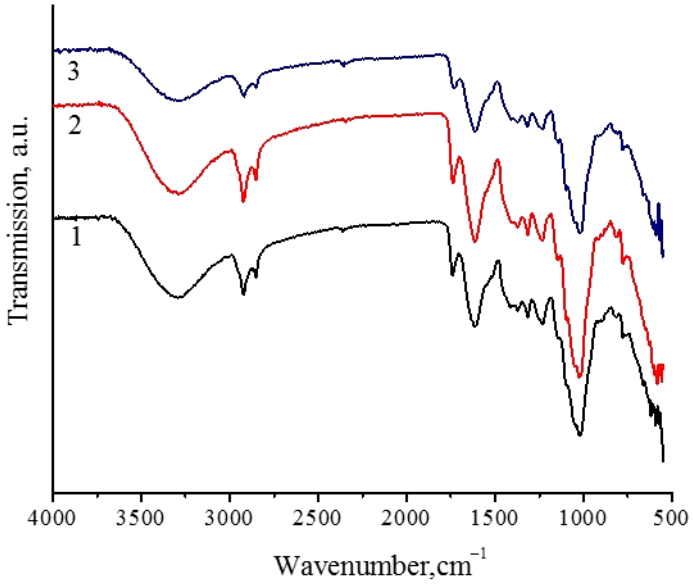


Fig. 5.3. FTIR specta of red hot pepper stalk with particle size of 0.25 mm (1), 0.5 mm (2) and 1.0 mm (3).

5.1.2. Surface color characteristics of red hot pepper fruit

CIE L*a*b values were determined of in different parts of the red hot pepper: pericarp, placenta, seed and stalk. The particle size of pericarp and stalk were with particle size of 0.25mm, 0.5 and 1 mm, where, particle size of placenta and seed was 0.5 and 1 mm. The appearance of the pericarp, placenta, seed and stalk of red hot pepper fruits with

different particle size is presented in Fig. 5.4, while in Table 5.5 are given surface color data determined.

The pericarp is characterized with the highest value of red colour (a*value) and chroma value (C) and brown index (BI) in comparison to the placenta, seed and stalk. In the pericarp by decreasing the particle size from 1 mm to 0.25 mm, the values of a*, C and BI increased and varied from 23.63 to 32.11 for a*, 30.55 to 41.94 for C and 80.99 to 108.43 for BI. The values for yellow color (b*) are the highest in seed (36.99). In the stalks in accordant to the particle size (1 mm to 0.25 mm), values of a*, b*, C and BI varied from 13.62-15.93; 26.52-29.69; 29.52-33.69 and 68.44-77.60, respectively (Table 5.5).

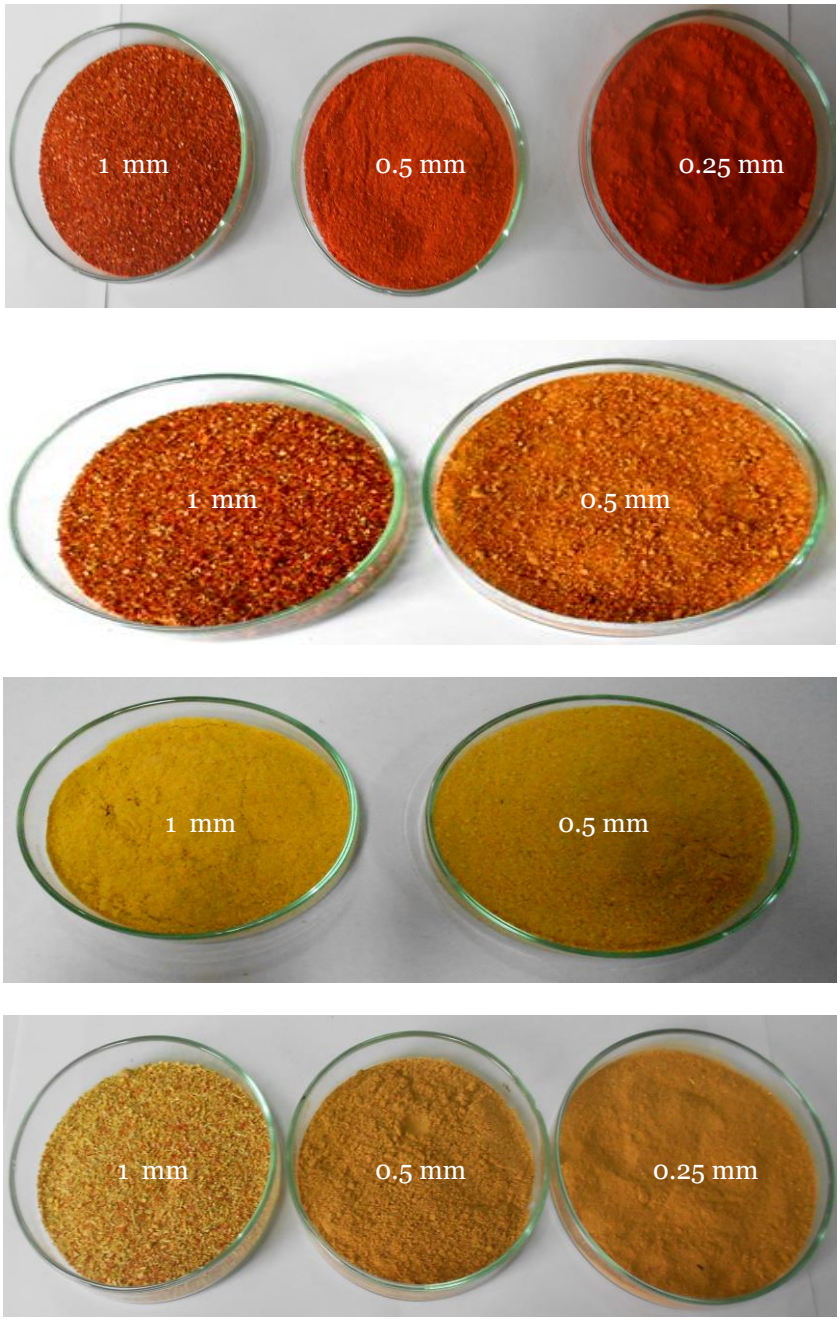


Fig. 5.4. Appearance of A) pericarp, B) placenta, C) seed and D) stalk of red hot pepper fruit with different particle size.

Table 5.5. Surface colour value of red hot pepper: pericarp, placenta, seed and stalk with different particle size.

	Particle size (mm)		Colour value L*	a*	b*	C	Ho	BI
Pericarp	0.25	$X_{average}$	54.24^{a}	32.11^{a}	26.99^{a}	41.94^{a}	35.02^{a}	108.43^{a}
		SD	0.22	0.48	0.67	0.78	0.17	2.67
		RSD (%)	0.40	1.5	2.45	1.86	0.48	2.47
	0.5	$X_{average}$	53.93^{b}	28.38^{b}	24.19^{b}	37.30^{b}	35.21^{a}	95.43^{b}
		SD	0.13	0.51	0.78	0.84	0.39	2.90
		RSD (%)	0.2432	1.7805	3.2040	2.24	1.12	3.04
	1	$X_{average}$	50.39^{c}	23.63^{c}	19.37^{c}	30.55^{c}	34.47^{a}	80.99^{c}
		SD	0.481	0.33	0.53	0.58	0.29	1.54
		RSD (%)	0.95	1.38	2.76	1.90	0.85	1.89
Placenta	0.5	$X_{average}$	65.57^{a}	24.97^{a}	32.39^{a}	40.90^{a}	42.43^{a}	93.83^{a}
		SD	0.09	0.23	0.27	0.21	0.23	0.74
		RSD (%)	0.14	0.93	0.84	0.50	0.53	0.79
	1	$X_{average}$	63.09^{b}	23.03^{b}	30.49^{b}	38.19^{b}	42.78^{a}	90.82^{b}
		SD	0.39	0.24	0.88	0.79	0.36	2.2
		RSD (%)	0.63	1.05	2.87	2.07	0.85	2.45
Seed	0.5	$X_{average}$	72.29^{a}	9.43^{a}	36.99^{a}	38.18^{a}	52.91^{a}	78.91^{a}
		SD	0.30	0.19	0.43	0.42	0.17	1.36
		RSD (%)	0.41	2.05	1.15	1.13	0.33	1.73
	1	$X_{average}$	70.32^{b}	9.36^{b}	36.85^{b}	38.02^{b}	52.90^{b}	81.40^{b}
		SD	0.49	0.36	0.5280	0.5573	0.47	1.55
		RSD (%)	0.69	3.79	1.43	1.47	0.88	1.91
Stalk	0.25	$X_{average}$	65.01^{a}	15.92^{a}	29.69^{a}	33.69^{a}	47.16^{a}	77.60^{a}
		SD	0.38	0.31	0.26	0.35	0.17	1.47
		RSD (%)	0.59	1.96	0.87	1.02	0.36	1.90
	0.5	$X_{average}$	63.89^{b}	14.57^{b}	27.41^{b}	31.04^{b}	47.26^{a}	71.65^{b}
		SD	0.56	0.20	0.38	0.37	0.16	0.47
		RSD (%)	0.87	1.38	1.39	1.24	0.35	0.65
	1	$X_{average}$	63.10^{b}	13.62^{c}	26.18^{c}	29.52^{c}	47.48^{a}	68.44^{c}
		SD	0.53	0.25	0.65	0.56	0.83	1.47
		RSD (%)	0.85	1.87	2.48	1.88	1.74`	2.15

$X_{average}$ – mean value of n=5; SD-standard deviation; RSD-relaltive standard deviation (%).^{a,b,c} Values with different superscripts within a column are significantly different (Tukey test, $p < 0.05$).

As presented in Table 5.5, surface color data of stalk samples ranged between of 64.29 to 68.42 (L*), 6.47 to 7.26 (a*) and 23.29 to 25.07 (b*). Color saturation (C*) and hue angle (Ho) decrease by increasing of particle size. The L* and Ho values significantly increase with decreasing stalk particle size ($p < 0.05$). In case of both solvents used, quantity of total carotenoids extracted from stalk samples increases by increasing particle size which is in agreement with the findings for a*. The stalk sample with 0.25 mm particle size was more dark, dull and deep yellow-brown in colour than samples with particle size of 0.5 and 1.0 mm. In literature no data were found about the colour characteristics of pepper stalk. The surface colour parameters published are related to the pericarp as an edible part of pepper fruits. The maturity degree of the pepper (Kim et al., 2008; Zaki et al., 2013), the influence of the drying and processing conditions (Gangadhar et al., 2012; Addala et al., 2015), as well as the differences in the pepper varieties were studied by using the characteristics of surface colour. The pericarp is characterized with lower values of L* (29.95÷31.61) and b* (6.99÷10.49). The a* values are higher and varied from 21.81 to 31.10 (Gangadhar et al., 2012; Zaki et al., 2013).

5.1.3. Volatile compounds of red hot pepper fruit

In the constituinal parts of red hot pepper fruits by using HS/GC and HS/GS/MS methods were analysed profile of volatile compounds.

Pericarp. In the pericarp of red pepper 38 volatile compounds were separated by HS/GC analysis. In Table 5.6 is shown the identity and quantity of nine components confirmed by using reference standards. Among all quantified volatiles, ester hexyl isobutanoate is high in quantity. The highest contents of hexyl isobutanoate (3.27 mg kg^{-1} DM) and tetradecanoic acid (3.05 mg kg^{-1} DM) were found in stalk with 1.0 mm particle size. The content of terpenoid *β*–ionone was almost six times higher in comparison to *α*–ionone.

Table 5.6. Volatile compounds (mg kg^{-1} DM)* in red hot pepper pericarp with different particle size.

Compound	Retention time (min)	Stalk particle size (mm)		
		0.25	0.5	1.0
Hexyl isobutanoate	15.413	0.33	0.21	0.24
Hexyl-*2*-methyl butanoate	19.429	0.53	0.187	0.17
Hexyl isopentanoate	19.540	0.17	0.08	0.04
Heptyl butanoate	20.934	0.86	0.12	0.36
Hexyl hexanoate	23.464	0.03	0.04	0.03
α–Ionone	24.805	0.03	0.01	0.01
β–Ionone	26.199	6.66	1.98	3.00
Tetradecanoic acid	32.386	0.23	0.47	0.02
Benzyl benzoate	32.777	0.05	0.01	0.01

The pericarp samples with particle size of 0.25 mm, 0.5 and 1.0 mm were anaysed by using HS/GS/MS method, also. The chromatograms of pericarp volatiles are shown in Fig. 5.5. From pericarp samples with 0.25 and 0.5 mm particle size 50 volatile compounds were separated, while with 1.0 mm particle size 100 volatile compounds. The volatiles were identified based on comparison of GC-MS NIST library according to mass spectra. In Table 5.7 are presented the volatile compounds separated from pericarp with area higher than 1% from total area of the separated peaks.

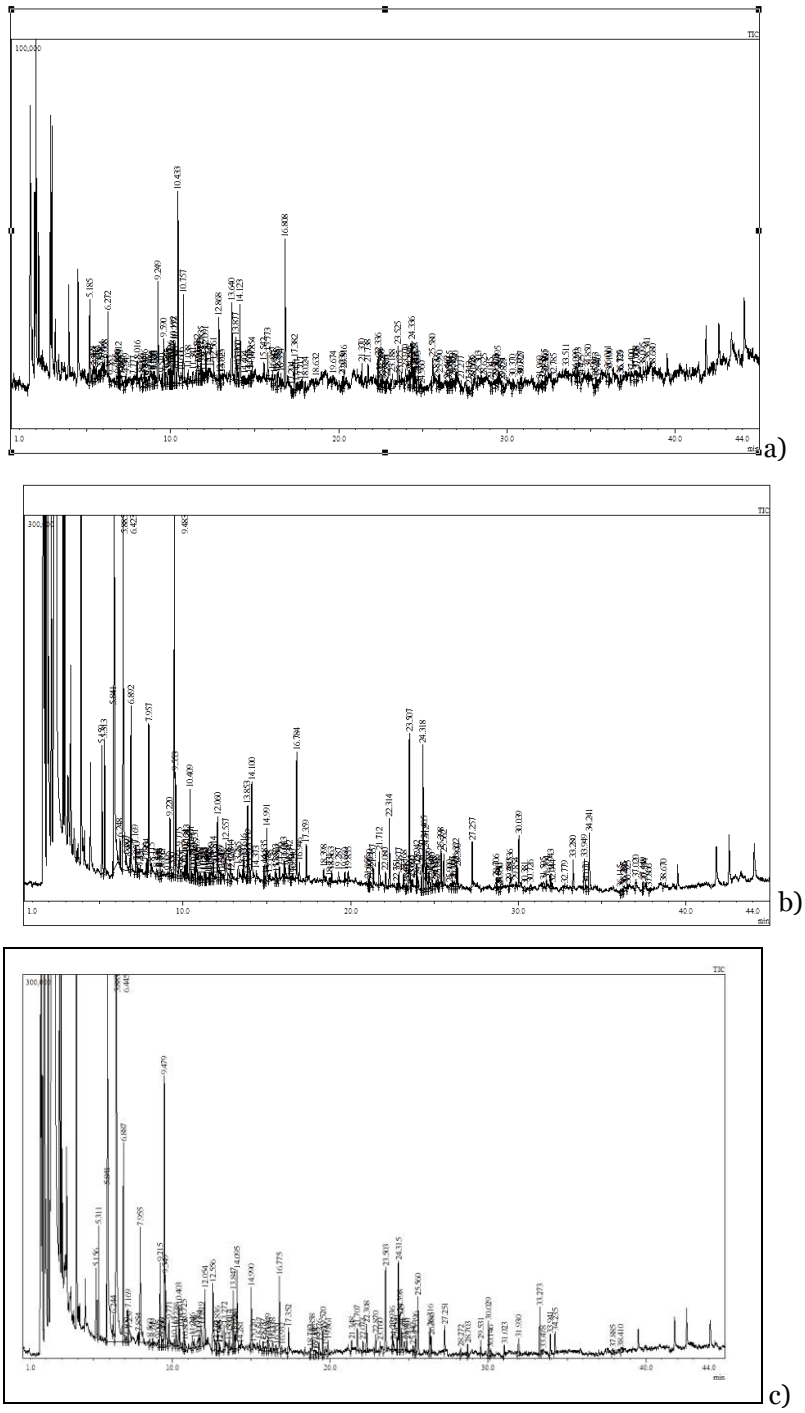


Fig. 5.5. HS/GS/MS chromatograms of volatiles of red hot pepper pericarp with particle size of 0.25 mm (a), 0.5 mm (b) and 1.0 mm (c).

In the pericarp with 0.25 mm and 0.5 mm particle size were separated 150 volatiles, from which 18 and 20, respectively were with area high than 1%. Twenty volatile compounds were with area higher than 2% from total 100 separated from the pericarp with the particle size of 1.0 mm.

Hexanal as alkyl aldehyde is separated from all pericarp samples. The area varied from 2.28 to 1.35% in the pericarp with 0.25 mm and 1.0 mm. Ketone 3(2H)-Furanone, dihydro-

2-methyl is determined in all analysed pericarp samples, with area from 1.85 to 2.33%. In the samples with high value of area (12.78-14.75%) was heterocyclic aldehyde furfural which is a colorless oily liquid with the odor of almond. Furfuryl alcohol or 2-furan methanol possesses a faint burning odor and a bitter taste. In the pericarp sampes the area of furfuryl alcohol increased from 7.44 to 13.63% by increasing the particle size from 0.25 to 1.0 mm. The area of the identified compound 2-furancarboxaldehyde, 5-methyl- in the pericarp samples increased from 5.95 to 8.21% by decreasing the particle size from 1.0 to 0.25 mm. Increasing the particle size of pericarp from 0.25 to 1.0 mm, increase the total area (62.28-69.07%) of the volatile compounds with area high that 1% (Table 5.7).

Table 5.7. Volatiles[1] of red hot pepper pericarp with different particle size.

No	Name*	Rt (min)	Area (%)		
			Particle size (mm)		
			0.25	0.5	1.0
1.	Hexanal	5.156	2.28	2.26	1.35
2.	3(2H)-Furanone, dihydro-2-methyl	5.313	1.85	2.33	2.13
3.	Ethane,1,2-bis[(4-amino-3-furazanyl)o	5.841	/	2.25	/
4.	Maleic anhydride	5.849	2.03	/	2.49
5.	Furfural	5.885	14.75	13.49	12.78
6.	2-Furan methanol	6.423	7.44	9.64	13.63
7.	*p*-Xylene	6.892	2.81	2.80	3.78
8.	Ethanone, 1-(2-furanyl)-	7.957	2.72	2.83	2.96
9.	2-Furanmethanol, 5-methyl-	9.215	/	/	2.08
10.	Bicyclo[3.1.0]hexan-3-ol, 4-methyl-1-(1-methylethyl)-, (1α,3β,4β,5α)-	9.220	/	1.21	/
11.	2-Furancarboxaldehyde, 5-methyl-	9.483	8.21	7.26	5.95
12.	Benzaldehyde	9.553	2.31	2.24	1.72
13.	2-Butanone, 1-(2-furanyl)	10.419	1.40	/	/
14.	Cyclohexanol, 4-(1,1-dimethylethyl)-trans	10.409	/	1.40	/
15.	Benzeneacetaldehyde	12.063	1.08	/	1.18
16.	Ethanone, 1-(1H-pyrrol-2-yl)-	12.556	/	/	2.21
17.	6-methyl-3,5-heptadiene-2-one	13.853	1.04	1.14	1.26
18.	Cyclohexanol, 2,6-dimethyl	14.100	1.30	1.98	1.97
19.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	14.991	3.17	1.17	1.45
20.	1,3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl-	16.775	2.02	/	1.96
21.	Tetradecane, 1-chloro-	16.784	/	2.54	2.00
22.	5,9-undecadien-2-one,6,10-dimethyl, (E)-	23.507	3.12	3.12	/
23.	3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	24.313	2.63	2.82	1.94
24.	3-Buten-2-one, 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)-	24.405	/	1.03	/
25.	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	25.560		/	1.45
26.	Methyl tetradecanoate	30.039	/	0.93	/
27.	Hexadecanoic acid, methyl ester	34.241	2.12	1.27	/
28.	Ethanone, 2,2-dimethoxy-1,2-diphenyl	33.173	/	/	1.07
	total		62.28	63.71	69.07

*identification based on GC-MS NIST library; 1-volatiles with area high than 1%

Stalk. In the stalk of red hot pepper 39 volatile compounds were separated by HS/GC analysis. The identity and quantity of nine components confirmed by using reference standards are listed in Table 5.8. Among all quantified volatiles, ester hexyl isobutanoate is high in quantity. The highest contents of hexyl isobutanoate (3.27 mg kg^{-1} DM) and tetradecanoic acid (3.05 mg kg^{-1} DM) were found in stalk with 1.0 mm particle size. The content of terpenoid *β*–ionone was almost six times higher in comparison to *α*–ionone. No data about the volatile compounds of pepper stalk were reported in the literature. Generally, the chili peppers varieties are well-known to be very aromatic and they contain characteristic volatile compounds. Hexyl isopentanoate and hexyl isobutanoate are responsible for powerful fruity odor note (Forero et al., 2009). *β*–ionone has great contribution as odour-active compound to pepper aroma according to Zimmermann and Schieberle (2000), while the presence of *α*–ionone, dihydro-*β*–ionone and *β*–ionone suggests that *β*–carotene may be considered to be its precursor (Pino, et al., 2011). Aromatic esters as benzyl pentanoate, hexyl benzoate and isohexyl benzoate have powerful fruity flavour notes and they might therefore contribute to the overall flavour of *Capsicum* species (Fernándes-Garciá et al., 2010).

Table 5.8. Volatile compounds (mg kg^{-1} DM)* in red hot pepper stalk with different particle size.

Compound	Retention time	Stalk particle size (mm)		
	(min)	0.25	0.5	1.0
Hexyl isobutanoate	15.417	1.38	1.78	3.27
Hexyl-2-methyl butanoate	19.426	0.02	0.02	0.18
Hexyl isopentanoate	19.523	0.20	0.12	0.10
Heptyl butanoate	20.904	0.32	0.28	0.09
Hexyl hexanoate	23.568	0.16	0.13	0.05
α–Ionone	24.695	0.51	0.39	0.29
β–Ionone	26.188	1.02	1.27	1.32
Tetradecanoic acid	32.386	0.02	0.02	3.05
Benzyl benzoate	32.777	1.02	0.09	0.03

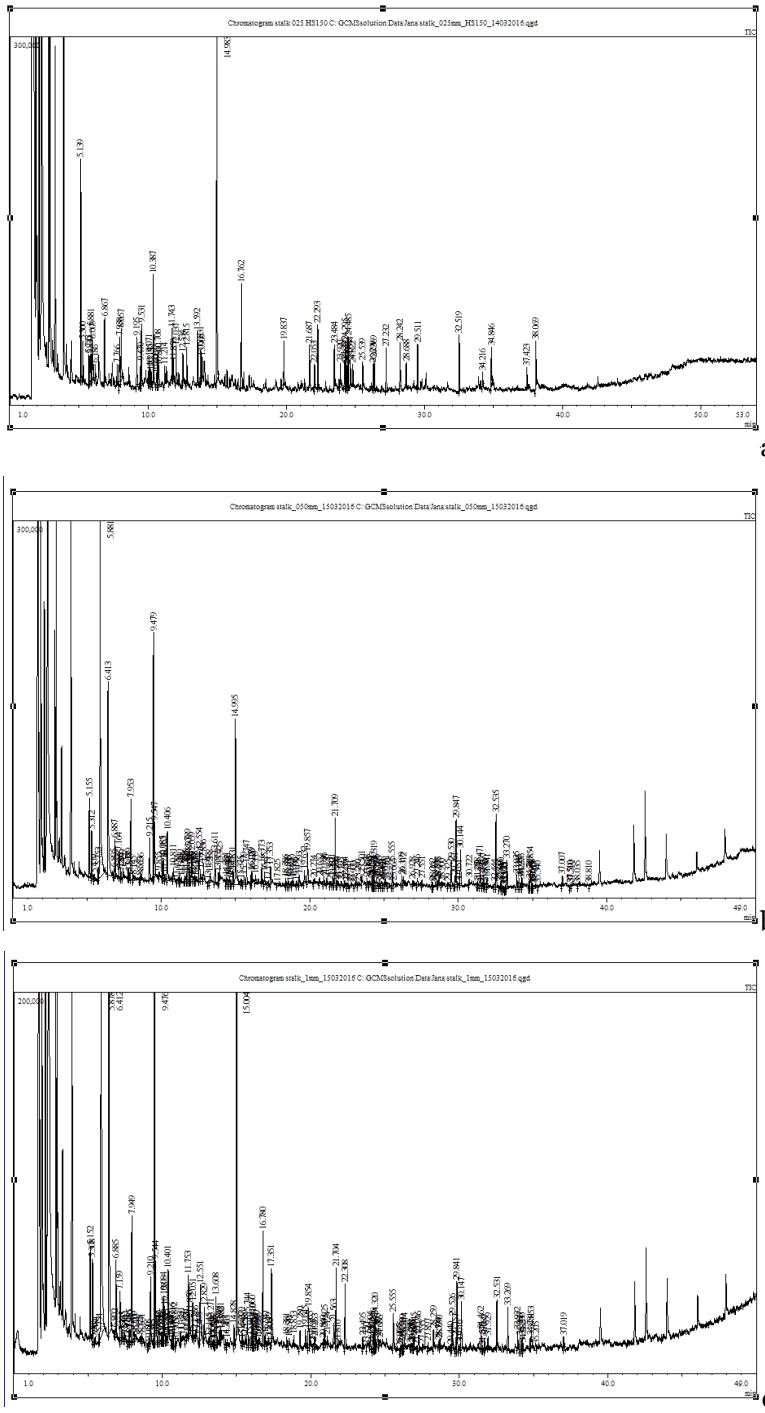


Fig. 5.6. HS/GS/MS chromatograms of volatiles of red hot pepper stalk with particle size of 0.25 mm (a), 0.5 mm (b) and 1.0 mm (c).

Table 5.9. Volatiles[1] of red hot pepper stalk with different particle size.

No	Name*	Rt (min)	Area (%) Particle size (mm)		
			0.25	0.5	1.0
1.	Hexanal	5.152	7.16	1.93	1.50
2.	3(2H)-Furanone, dihydro-2-methyl	5.275	1.31	1.34	1.46
3.	Pyrazine, methyl-	5.733	1.26	/	/
4.	Ethane,1,2-bis[(4-amino-3-furazanyl)o	5.830	1.15	/	/
5.	Furfural	5.878	2.62	17.59	18.08
6.	1-pentanol 4-methyl	6.009	1.60	/	/
7.	2-Furan methanol	6.412	/	7.27	8.44
8.	*o*-Xylene	6.885	2.70	1.15	1.31
9.	4-Cyclopentene-1,3-dione	7.159	/	1.18	1.38
10.	Ethanone, 1-(2-furanyl)-	7.949	2.15	2.67	2.53
11.	Pyrazine,26-dimethyl	8.057	2.04	/	/
12.	(1S,4S,5R)-1-Isopropyl-4-methylbicyclo[3.1.0]hexan-3-one (β-thujone)	9.195	1.85	/	/
13.	2-Furanmethanol, 5-methyl-	9.215	/	6.99	/
14.	Bicyclo[3.1.0]hexan-3-ol, 4-methyl-1-(1-methylethyl)-, (1α,3β,4β,5α)-	9.210	/	1.21	1.38
15.	2-Furancarboxaldehyde, 5-methyl-	9.479	/	6.99	7.40
16.	Benzaldehyde	9.531	2.47	1.74	1.80
17.	Decane, 2,2-dimethyl-	10.409	/	/	1.04
18.	Cyclohexanol, 4-(1,1-dimethylethyl)-trans	10.406	3.47	1.58	/
19.	Undecane	10.708	1.29	/	/
20.	Octanal	10.800	1.13		
21.	2-Pyrrolidine-, 1-methyl	11.753	2.87	1.47	1.68
22.	Benzeneacetaldehyde	12.037	1.36	1.00	1.18
23.	Ethanone, 1-(1H-pyrrol-2-yl)-	12.534	1.16	1.10	1.37
24.	Benzene, (1,1-dimethylethoxy)-	12.815	1.34	/	/
25.	Benzoic acid, methyl ester	13.592	1.85		1.03
26.	6-methyl-3,5-heptadiene-2-one	13.790	1.21	/	/
27.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	15.004	15.38	6.10	9.30
28.	Hexadecane,1-chloro	16.762	4.14	/	
29.	Dodecane	16.780	/	/	2.15
30.	5-Hydroxymethylfurfural	17.351	/	/	1.70
31.	2-Metoxy-4-vinylphenol	19.837	1.61	1.02	/
32.	2-Propenoic acid 1 7 7-trimethylbicyclo 2.2.1 hept-2-yl ester	21.704	1.42	2.18	1.51
33.	Tetradecane	22.308	2.21	/	1.22
34.	5,9-undecadien-2-one,6,10-dimethyl, (E)-	23.484	1.54	/	/
35.	trans-β-ionone	24.295	1.87	/	/
36.	(4R,4aS,6S)-4,4a-Dimethyl-6-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene	24.485	2.05	/	/
37.	Heneicosane	26.369	1.10	/	/
38.	Octadecane	27.232	1.52	/	
39.	N-Propyl-9-borabicyclo[3.3.1]nonan-9-amine	28.242	1.88	/	/
40.	Heptadecane	29.465	1.58	/	
41.	Acetic acid, 3- hydroxyl-6-isopropenyl-4	29.841	/	2.26	1.49
42.	Methyl tetradecanoate	30.039	/	0.93	/
43.	6-Methyl-2-(4-methyl-3-cyclohexen-1-yl)-5-hepten-2-ol (bisabolol)	30.144		1.29	1.05
44.	2-Pentadecanone,6,10,14-trimethyl	32.535	2.19	2.21	/
45.	Hexadecanoic acid, methyl ester	34.241	/	1.27	/
46.	Ethanone, 2,2-dimethoxy-1,2-diphenyl	33.270	/	1.09	/
	total		82.67	73.56	70.00

*identification based on GC-MS NIST library; 1-volatiles with area high than 1%

5.2. INFLUENCE OF THE FACTORS AT EXTRACTION OF RED HOT PEPPER

5.2.1. Application of conventional methods

5.2.1.1. Extraction of red hot pepper by maceration

The influence of the working parameters: time, temperature, and solid to liquid ratio was followed at extraction of the placenta from red hot pepper with 0.25 mm particle size according the conditions for the central composite design given in Table 4.1. A three-predictor nonlinear regression model was used to evaluate the individual and interactive effects of three-independent variables: time (x1), temperature (x2) and solid to liquid phase ratio (x3). The responses measured were yield of extract, capsaicin and major pigment capsanthin in the obtained extract. The second order model includes linear, quadratic and interactive terms thus, in the responses function (Y)-Eq. 5.1, xi and xj are predictors; β0 is the intercept; βi are linear coefficients; βii are squared coefficients; βij are interaction coefficients and ε is an error term. ANOVA was used to evaluate the significances of the coefficients of the models judged by computing the F-value at a probability (p) of 0.001, 0.01 and 0.05. The values of the obtained quantities of the extract are presented in Table 5.10.

$$Y = b_0 + \sum_{i=1}^{k} b_i x_1 + \sum_{i=1}^{k} b_{ii} x_2 + \sum_{i=1}^{k} b_{ii} x_3 + \sum_{i>1}^{k} b_{ij} x_i x_j x_z + \varepsilon \qquad (5.1.)$$

Table 5.10. Yield of extract obtained at extraction of placenta of red hot pepper with ethanol.

No of experiment	Time - X_1 (min)	Temperature - X_2 (^oC)	Solid : liquid - X_3 (g/ cm^3)	Yield of extract (%)
1	60	20	0.1	26.0122
2	300	20	0.1	24.9619
3	60	60	0.1	24.3236
4	300	60	0.1	22.8979
5	60	20	0.025	33.1212
6	300	20	0.025	28.4501
7	60	60	0.025	29.1086
8	300	60	0.025	26.4224
9	60	40	0.04	33.0479
10	180	20	0.04	21.7800
11	180	40	0.1	34.1070
12	300	40	0.04	30.1014
13	180	60	0.04	24.1118
14	180	40	0.25	34.4494
15	180	40	0.04	33.4664
16	180	40	0.04	32.9736
17	180	40	0.04	34.0497
18	180	40	0.04	34.0704
19	180	40	0.04	34.7261

Yield of extract. The values of the obtained quantities of the extract are presented in Table 5.10, while Table 5.11 shows the liner, quadratic and interactive coefficients of the independent variables in the models and their corresponding R^2 when ethanol was used as extraction solvent of the placenta of hot red pepper.

Table 5.11. Regression coefficients, R^2, adjusted R^2 and *p* for three dependent variables for extract of placenta of red hot pepper.

	Yield of extract (%)
b_0 (interpect)	24,886***
b_1	- 0,056
b_2	1,401***
b_3	- 564,673**
b_1^2	0,0000
b_2^2	- 0,018***
b_3^2	3978,741**
b_{12}	0,0000
b_{23}	0,133
b_{13}	- 0,001
R^2	0,9276
adjusted R^2	0,8552
p	0,0004

Subscripts: 1= time (min); 2 = temperature (°C) ; 3 = solid and liquid ratio (g/cm^3)
*Significant at 0.05 level; **Significant at 0.0l level; ***Significant at 0.001 level.

At extraction of placenta from hot red pepper, on the quantity of obtained extract statistically is significant the influence of the temperature and the solid to liquid phase ratio. For influence of the extraction temperature, the value of linear coefficient (b_2=1.401) and quadratic term (b_2^2=-0.018) were determined at 0.001 significant level. By increasing the solid to liquid ratio smaller quantities of extract were obtained (b_3= - 564,673). The R^2 values for these response variables are higher than 0.9276, indicating that the regression models adequately explained the process. In Fig. 5.7 are presented the contour plot for the influence of time, temperature and ratio of solid and liquid phase on the quantity of extract of red hot placenta.

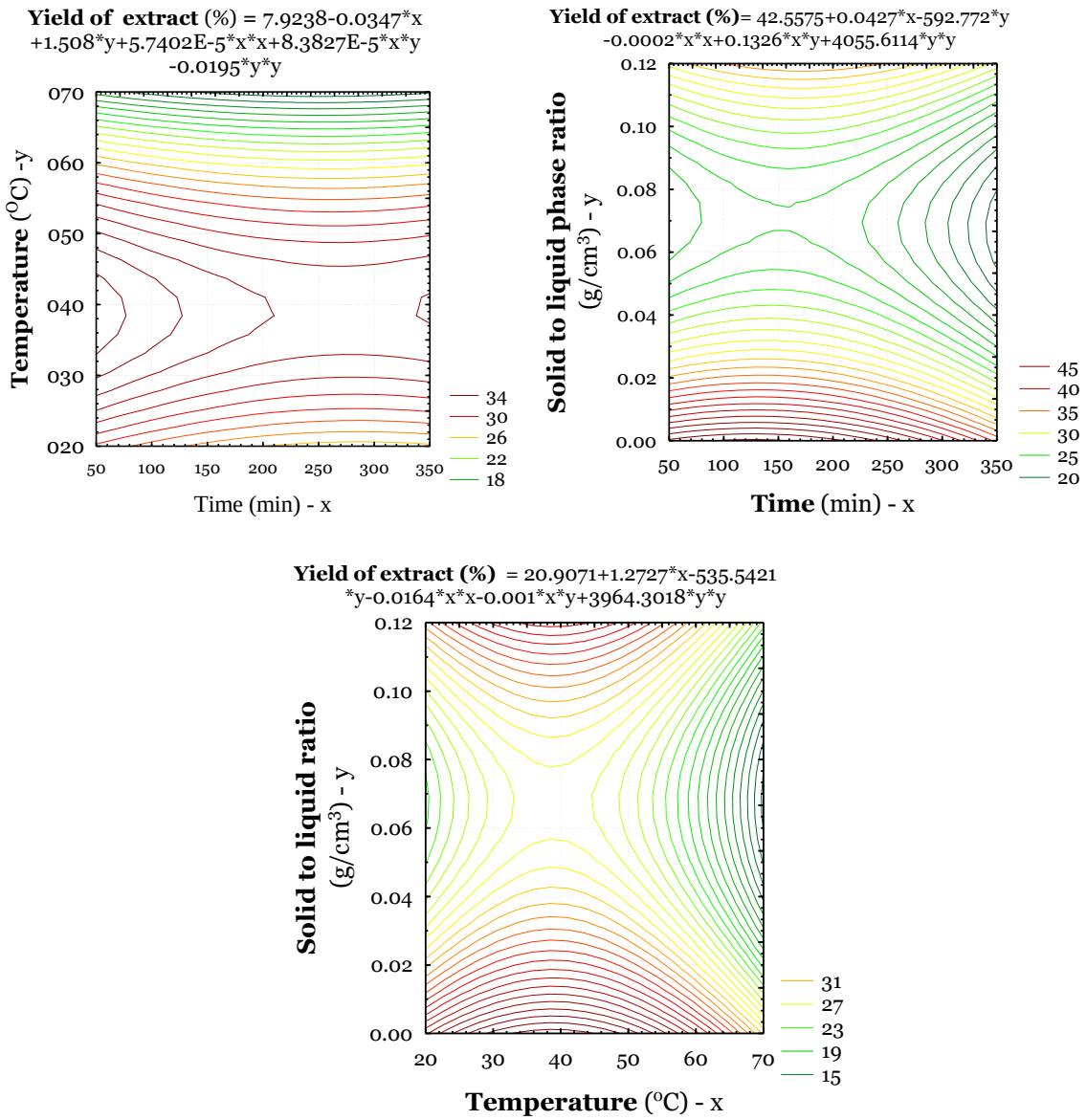


Fig. 5.7. Contour plot for the influence of time, temperature and ratio of solid and liquid phase on the quantity of extract from red hot placenta.

Capsaicin extraction. In Table 5.12 are given the liner, quadratic and interactive coefficients of the independent variables and their corresponding R^2 in the model for extraction of capsaicin from placenta of red hot pepper when ethanol was used. The calculated linear and quadratic coefficients were b_3= 1042.64 и b_3^2 = -7977.87, while the regression coefficient (R^2) was 0.9108. The optimal value for the quantity of extracted capsaicin (27.98 mg/100 g extract) from placenta of red hot pepper was obtained at 170 min, 55°C and solid to liquid ratio of 0.0478 g/cm³. The influence of time, temperature and ratio of solid and liquid phase on the quantity of capsaicin in the placenta extract of red hot pepper was presented by the contour plots, also (Fig. 5.8).

Table 5.12. Regression coefficients, R^2, adjusted R^2 and *p* for three dependent variables for capsaicin quantity in extract of placenta of red hot pepper.

	Capsaicin in extract (mg/100 g)
bo (interpect)	-16.95***
b1	0.11
b2	0.05
b3	1042.64***
b12	0,0000
b22	0.00
b32	-7977.87***
b12	-0,0000
b23	0.18
b13	- 0,91
R^2	0,9108
adjusted R^2	0,8216
p	0,0009

Subscripts: 1= time (min); 2 = temperature (°C) ; 3 = solid and liquid ratio (g/cm³)
*Significant at 0.05 level; **Significant at 0.0l level; ***Significant at 0.001 level.

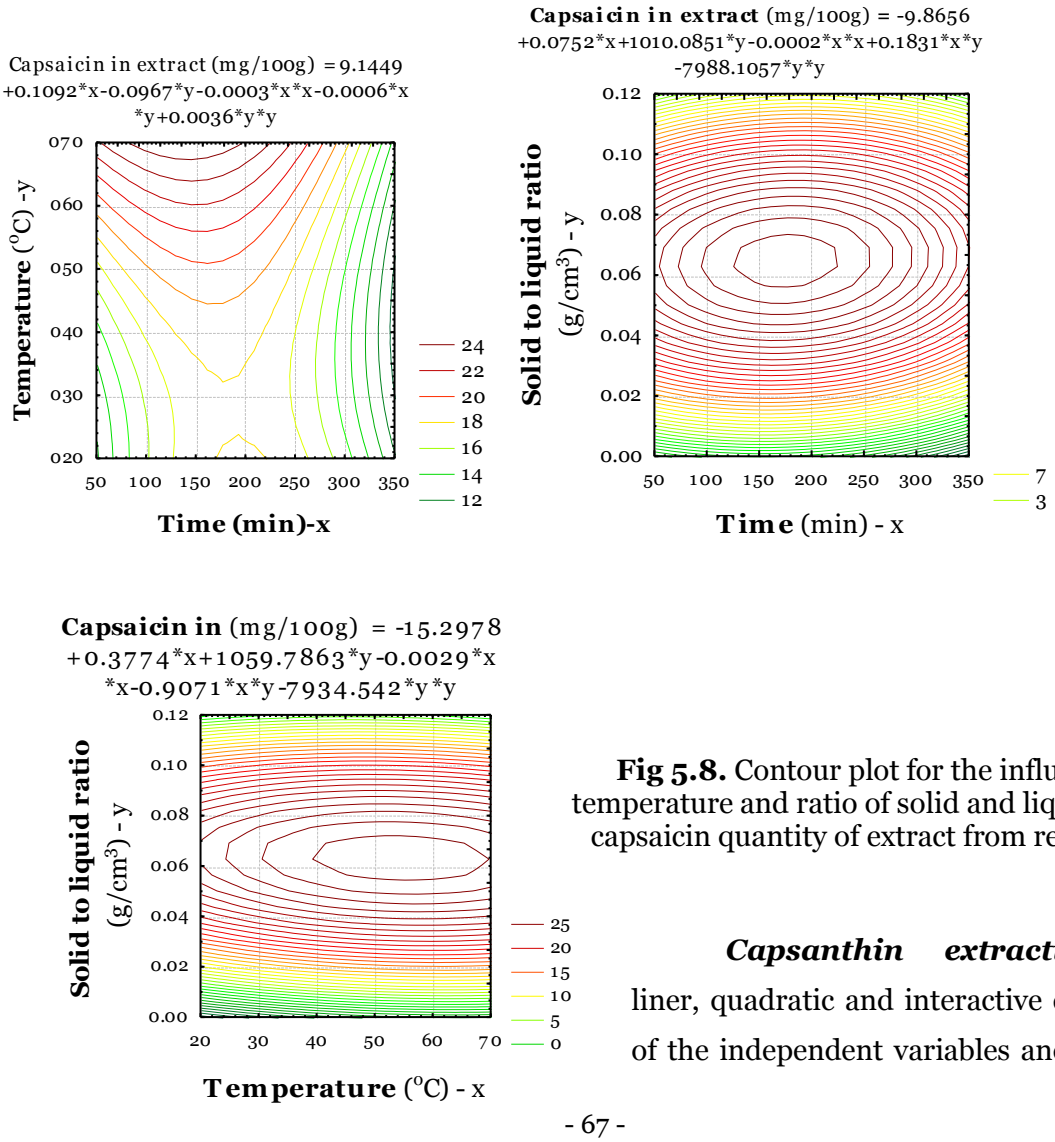


Fig 5.8. Contour plot for the influence of time, temperature and ratio of solid and liquid phase on the capsaicin quantity of extract from red hot placenta.

Capsanthin extraction. The liner, quadratic and interactive coefficients of the independent variables and R^2 in the

model for extraction of capsanthin from placenta of red hot pepper with ethanol are given in Table 5.9. The R^2 value for the model developed for describing the process of extraction of capsanthin from placenta of red hot pepper was 0.73, which was confirmed that developed mathematical model was unappropriated in the description of the extraction process of capsanthin from red hot pepper (Joglekar and May, 1977; Montgomery, 2001).

Table 5.13. Regression coefficients, R^2, adjusted R^2 and p for three dependent variables for capsanthin quantity in extract of placenta of red hot pepper.

	Capsanthin in extract (mg/100 g)
b_0 (itercept)	164.8
b_1	2.1
b_2	6.1
b_3	15135
b_1^2	0,0000
b_2^2	0.00
b_3^2	-52001.9
b_{12}	-0,0000
b_{23}	26.3**
b_{13}	- 3.1
R^2	0.7300
adjusted R^2	0.4601
p	0.0772

Subscripts: 1= time (min); 2 = temperature (°C) ; 3 = solid and liquid ratio (g/cm^3)
*Significant at 0.05 level; **Significant at 0.0l level; ***Significant at 0.001 level.

The contour plots obtained at study the influence of time, temperature and ratio of solid and liquid phase on the quantity of capsantin in the placenta extract of red hot pepper are presented in Fig. 5.9.

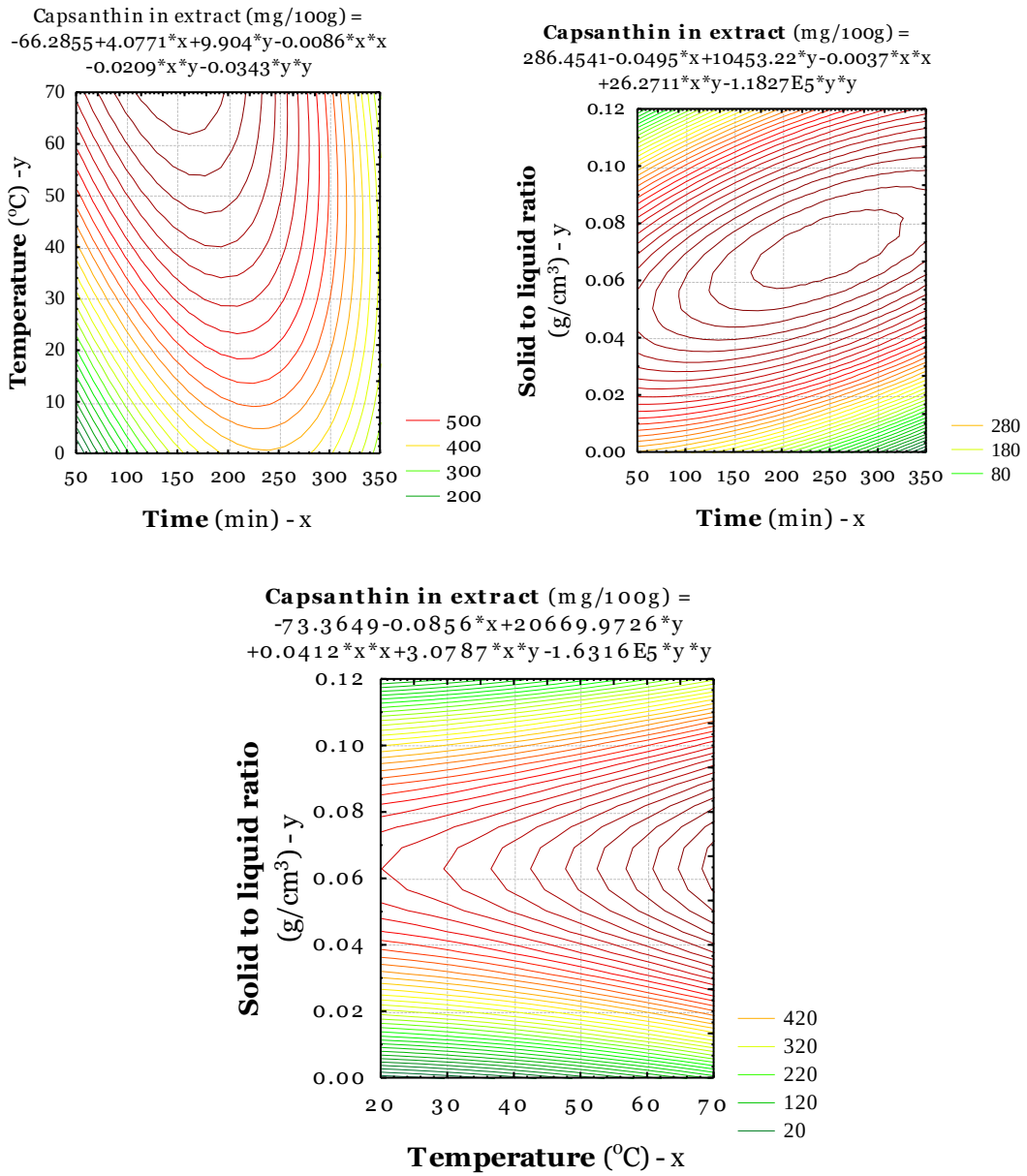


Fig 5.9. Contour plot for the influence of time, temperature and ratio of solid and liquid phase on the capsanthin quantity in extract from red hot placenta.

Summary for the extraction of placenta of hot red pepper by maceration. In the process of solid-liquid extraction of the placenta from the red hot pepper with ethanol using a technique of maceration in a thermostatic water bath, to obtain the satisfied quantity of extract with high amounts of capsaicin and capsanthin, the extraction at temperature of 40 °C to 50 °C, time of 180 min and 0.04 g /cm^3 solid and liquid phase ratio is is recommended.

5.2.1.2. Extraction of red hot pepper by ultrasound

At extraction of pericarp (0.25 mm particle size) from red hot pepper with ultrasound, the influence of the working parameters: time, temperature, solid to liquid ratio and type of solvent (ethanol and *n*-hexane) was studied. The values of the parameters used in the extraction process are given in Table 4.2.

Yield of extract. The quantities of extract obtained by extraction of pericarp with ethanol and *n*-hexane at ultrasound conditions are given in Table 5.14. Compared to *n*-hexane, with ethanol as extraction solvent high extract quantities were obtained. The highest extract quantity (41%) with ethanol was obtained at 70°C, 5 min and 0.01 g/cm^3 solid to liquid phase ratio, while with *n*-hexane (26.5%) at 20°C, 30 min and 0.01 g/cm^3 solid to liquid phase ratio.

Table 5.14. Yield of extract from pericarp of red hot pepper obtained with ethanol (Y_A) and *n*-hexane (Y_B) at ultrasound extraction.

No. of experiment	Temperature - X_1 (°C)	Time - X_2 (min)	Solid to liquid phase - X_3 (g/cm^3)	Yield of extract –Y_A (%)	Yield of extract – Y_b (%)
1	20	5	0.05	30.6232	20.9158
2	20	5	0.05	29.8561	21.4714
3	70	5	0.05	36.7926	4.4391
4	70	5	0.05	38.4769	4.9600
5	20	30	0.05	26.9584	21.4614
6	20	30	0.05	26.5440	20.9274
7	70	30	0.05	38.0571	4.2566
8	70	30	0.05	37.6823	3.9800
9	20	5	0.01	34.3319	21.0000
10	20	5	0.01	36.5180	20.8200
11	**70**	**5**	**0.01**	**40.9354**	5.4912
12	**70**	**5**	**0.01**	**40.8954**	5.4545
13	**20**	**30**	**0.01**	36.6453	**26.3547**
14	**20**	**30**	**0.01**	37.7498	**26.3600**
15	70	30	0.01	44.4511	6.6373
16	70	30	0.01	44.8193	5.8800

The values of the regression coefficients obtained by application of response surface methodology to establish mathematical models to predict system responses in the extraction of pericarp with ethanol and *n*-hexane at ultrasound are presented in Table 5.15. At extraction of the pericarp with *ethanol* in ultrasound water bath, the temperature and the solid to liquid phase ratio significantly influenced the yield of extract. For the temperature as independent variable, the value of linear coefficient (b_1=0.094) was determined at 0.01 significant level. By decreasing the solid to liquid ratio extract (b_3= -118.668), the quantity of increased. The model used well fitted the extraction of pericarp with ethanol that was

confirmed by the values for the R^2 (0.9891) and confidential level ($p < 0.0001$). In application of *n*-hexane for extraction of pericarp, the influence of the temperature and time is significant. For the temperature, the value of the linear coefficient ($b_{1=}$-0.3125) was determined at 0.001 confidential level. By increasing the time of extraction, increase the quantity of extract (b_2= 0.1671). The value of correlation coefficient (R^2) was 0.9914.

Table 5.15. Regression coefficients, R^2, adjusted R^2 and *p* for quantity of pericarp extract from red hot pepper obtained with ethanol (Y_A) and *n*-hexane (Y_B) at ultrasound extraction.

	Yield of extract –Y_A (%)	Yield of extract –Y_B (%)
b_0 (intercept)	34.219***	26,7745***
b_1	0.094**	-0,3125***
b_2	0.099	0,1671*
b_3	- 118.668**	6,29853
b_{12}	0.001	-0,00130
b_{23}	0.7733	-2,40536
b_{13}	- 5.969**	-0,00855
b_{123}	0.036	-
b_1^2	-	-
b_2^2	-	-
b_3^2	-	-
R^2	0.9891	0,9914
adjusted R^2	0.9796	0,9858
p	0.0000	0,0000

Subscripts: 1 = temperature (°C); 2 = time (min); 3 = solid and liquid ratio (g/cm^3)
*Significant at 0.05 level; **Significant at 0.0l level; ***Significant at 0.001 level.

Capsaicin and dihydrocapsaicin in extract. In Table 5.16 are shown the regression coefficient values determined for the influence of the independent variables (time, temperature and solid to liquid ratio) at ultrasound extraction of capsaicin and dihydrocapsaicin from red hot pepper pericarp using the ethanol and *n*-hexane as solvents. The temperature (b_1=-0.0649) and solid to liquid phase (b_3=-46.5755) influenced the content of capsaicin and dihydrocapsaicin in the pericarp extracts obtained with ethanol, while in case of *n*-hexane the influence of the temperature was significant (b_1= 1.358). The regression coefficients obtained in the modelling of the pericarp extraction with ethanol and *n*-hexane were 0.9133 and 0.9926, respectively.

Table 5.16. Regression coefficients, R^2, adjusted R^2 and *p* for quantity capsaicin and dihydrocapsaicin (CAP + DHC) in pericarp extract from red hot pepper obtained with ethanol (Y_A) and *n*-hexane (Y_B).

	CAP + DHC in extract –Y_A (mg/g)	CAP + DHC in extract –Y_B (mg/g)
b_0 (intercept)	16,1633***	-8,7327**
b_1	-0,0649**	1,4549***
b_2	-0,0101**	0,0136**
b_3	- 46,5755	34,7869**
b_{12}	-	-0,0073*
b_{23}	-	-12,2758**
b_{13}	-	-1,7022**
b_{123}	-	0,1934
b_1^2	-	-
b_2^2	-	-
b_3^2	-	-
R^2	0,9133	0,9944
adjusted R^2	0,8483	0,9895
p	0,0000	0,0000

Subscripts: 1 = temperature (°C); 2 = time (min); 3 = solid and liquid ratio (g/cm³)
*Significant at 0.05 level; **Significant at 0.0l level; ***Significant at 0.001 level.

Colored parameters of the extracts obtained from pericarp at extraction with ethanol and *n*-hexane are presented in Table 5.17.

Table 5.17. Colored parameters of extracts from pericarp of red hot pepper obtained at ultrasound with ethanol and *n*-hexane.

No of experiment*	Color parameter				
	L*	a*	b*	Ho	C
ethanol					
1	38.41±0.36	6.58±0.19	6.03±0.29	42.50±0,03	8.93±0.26
2	40.06±0.03	6.11±0.1	7.88±0,22	52.21±0,02	9.98±0.17
3	38.34±0.21	5.02±0.32	4.59±0.42	42.44±0.04	6.81±0.42
4	24.50±0,12	10.79±0.2	14.63±0.35	53.59±0.01	18.19±0.3
5	38.82±0.06	5.22±0.11	5.42±0.3	46.07±0.04	7.54±0.18
6	26.72±0.13	7.67±0.16	15.97±0.41	64.35±0.41	17.72±0.35
7	41.31±0.05	4.00±0.11	8.88±0,22	65.75±0.02	9.75±0.16
8	41.25±0.05	4,.08±0.07	9±0,18	65.61±0.01	9.88±0.15
n-hexane					
1	26.86±0.15	7.47±0.14	9.61±0.71	52.14±0.04	12.2±0.53
2	26.57±0.41	8.25±0.28	6.97±0.47	40.19±0.04	10.8±0.39
3	26.22±0.10	7.91±0.26	7.09±0,88	41.,87±0.07	10.64±0.57
4	26.17±0.09	8.06±0.26	6.64±0.64	39.48±0.06	10.47±0.29
5	25.85±0.37	7.46±0.5	5.85±1.04	38.10±0.07	9.51±0.29
6	25.25±0.16	7.41±0.28	5.3±0.58	35.57±0.07	9.14±0.83
7	25.38±0.11	7.36±0.12	5.63±0.19	37.41±0.02	9.27±0.15
8	26.06±0.08	7.61±0.17	5.31±0.26	34.91±0.02	9.28±0.23

* number according to Table 4.2.

In general, taking in matter the nonpolar characteristics of coloured compounds in red hot pepper and n-hexane, the pericarp extract obtained at ultrasound extraction with *n*-hexane are with higher value for a* (7.36 to 8.25), while ethanolic extract are characterized with higher values for color parameter b* as a measure of the yellow/blue colour. The differences in the colour (ΔE) estimated between the conditions at extraction are higher than 5, and varied for 6.92 to 7.35 for ethanol extracts, while for *n*-hexane extracts the colour differences ranged from 6.39 to 6.62.

Summary for the extraction of pericarp of hot red pepper by ultrasound. The results obtained at the study of the influence of the working parameters: temperature, time and solid to liquid ratio show to performed extraction at higher temperatures for short time. The biggest quantity of extract and capsaicinoids were obtained at 70 °C, 5 min and 0.01 g/cm^3 solid to liquid ratio by application of the ethanol as solvent, while with *n*-hexane at 20 °C, 5 min and 0.01 g/cm^3. The influence of the temperature and time is significant for the extraction of coloured compounds, while the influence of ratio of the solid to liquid phase is insignificant. Due to the degradation of the coloured compounds at higher temperature, extraction to max 60 °C and 30 min is recommended.

5.2.1.3. Extraction of red hot pepper by *Soxhlet* method

The influence of the particle size (0.25 mm, 0.5 mm and 1.0 mm) at extraction of pericarp, placenta, seed and stalk from red hot pepper by applying the Soxhlet method with petroleum ether (40-60°C) was followed by determination of the total quantity of extract, as well as the quantity of capsaicin and coloured compounds in extracts. During the extraction of 420 min, in each cycle from total seven cycles, yield of extract was determined. Total quantity of extract was estimated as a sum of extract obtained at seven cycles and at the end of the extraction, after 420 min.

The yields of extracts is presented in Table 5.18. At extraction of pericarp, the highest quantity of extract (6.42%) was obtained from the pericarp with 0.25 mm particle size. Increasing the particle size from 0.5 to 1.00, quantity of extract deceased from 4.60 to 2.68%. During the placenta extraction with particle size of 0.5 and 1.0 mm, yield of extract of 7 and 7.05%, respectively, were obtained. Compared to pericarp, placenta and stalk, from seed the highest amount of extract was obtained. At extraction of seed with 1.0 mm particle the yield of extract (16.67%) was 5.65% smaller compared with the seed extract obtained at

0.5 mm particle size. The yields of extracts obtained at stalk extraction with particle size of 0.25 mm. 0.5 and 1.0mm were 3.70%, 2.99 and 1.98%, respectively.

Table 5.18. Quantity of extract from hot red pepper obtained with petroleum (40-60°C), 420 min time of extraction.

Sample	Particle size (nm)	Yield of extract (%)	
		1*	2*
pericarp	0.25	6.13	6.42
	0.50	4.35	4.6
	1,00	2.55	2.68
placenta	0.50	6.07	7.05
	1.00	5.28	5.59
seed	0.50	21.26	22.32
	1.00	15.9	16.67
stalk	0.25	3.42	3.70
	0.50	2.79	2.99
	1.00	1.87	1.98

*1-extract obtained at 420 time of the extraction; *2-total extract = 1* + extract obtained at extraction for 7 cycles.

In Table 5.19., estimated equations and parameters of the empirical models for quantity of extract of pericarp, placenta, seed and stalk obtained with petroleum ether (40-60°C) for 420 min extraction are presented. The empirical models developed show well-fitting with the experimental obtained data for the yield of extract. High values of correlation coefficient were obtained for all applied models. The values for R^2 ranged from 0.8638 to 0.9934.

Table 5.19. Equations and parameters for empirical models developed for the quantity of extract obtained by extraction of pericarp, placenta, seed and stalk with petroleum ether (40-60°C).

Model	$Y = t/(k_1+t)$; $Y=e_1[1-\exp(-k_1 \cdot t)]+k_2 \cdot t$;		$Y = k_1 \cdot t/(k_2+t)$ $Y=1-k_1\exp(-k_2 * t)-k_3\exp(-k_4 \cdot t)$				
Sample	**Paericle size** (mm)	k_1	k_2	k_3	k_4	e_1	R^2
перикарп	0.25	55.5055	–	–	–	–	0.9898
		1.0657	70.6165	–	–	–	0.9927
		0.0187	0.0006	-	-	0.7210	0.9949
		0.9784	0.0010	0.0003	0.1122	–	0.9818
	0,.50	83.5617	–	–	–	–	0.9198
		1.0957	110.9436	–	–	–	0.9245
		0.2506	0.0016	–	–	0.3392	0.9831
		0.9821	0.0109	0.0001	0.1117	–	0.8852
	1.00	36.3222	–	–	–	–	0.9928
		1.0162	39.2311				0.9931
		0.2488	0.0009			0.6224	0.9644
		0.9638	0.0133	0.0003	0.1139	–	0.9705
плацента	0.50	80.5265	–	–	–	–	0.8638
		0.0028	356.015	–	–	–	0.9927
		0.2487	0.0023	–	–	0.1914	0.8654
		1.0778	0.0006	-0.003	0.0129	–	0.9459
	1.00	74.3116	–	–	–	–	0.9007
		1.1040	100.8680	–	–	–	0.9069
		0.2488	0.0015	–	–	0.3766	0.9666
		0.8991	0.0058	$-5.7 \cdot 10^{-6}$	-0.7730	–	0.8879
семе	0,50	53.4017	–	–	–	–	0.9553
		1.1330	119.8418	–	–	–	0.9923
		0.2487	0.0019	–	–	0.3773	0,9518
		0.9974	0.0092	$-2.3 \cdot 10^{-5}$	-0.7750	–	0.9934
	1,00	58.5010	–	–	–	–	0.9729
		1.1208	104.6518	–	–	–	0.9915
		0.2487	0.0013	–	–	0.4132	0.9848
		0.9736	0.0085	$3.6 \cdot 10^{-5}$	-0.7749	–	0.9827
дршка	0,25	28,4948	–	–	–	–	0.9733
		1,0840	41.9599	–	–	–	0.9804
		0,2471	0.0011	–	–	0.6132	0.9649
		0,9684	0.0149	$1.8 \cdot 10^{-5}$	-1.8446	–	0.9627
	0,50	53.4017	–	–	–	–	0.9553
		1.1330	119.8418	–	–	–	0.9923
		0.2487	0.0019	–	–	0.3773	0.9518
		0.9974	0.0092	$-2.3 \cdot 10^{-5}$	-0.7750	–	0.9934
	1,00	101.3408	–	–	–	–	0.9012
		2.7238	678.5315	–	–	–	0.9919
		0.0014	- 0.0019	–	–	4.1325	0.9930
		1.0012	0.0033	$- 9.8 \cdot 10^{-5}$	-1.9498	–	0.9920

After the extraction, on the extracted raw material colorimetric characteristics were determined. The appearance of the extracted samples of pericarp, placenta, seed and stalks in Fig. 10 is presented.

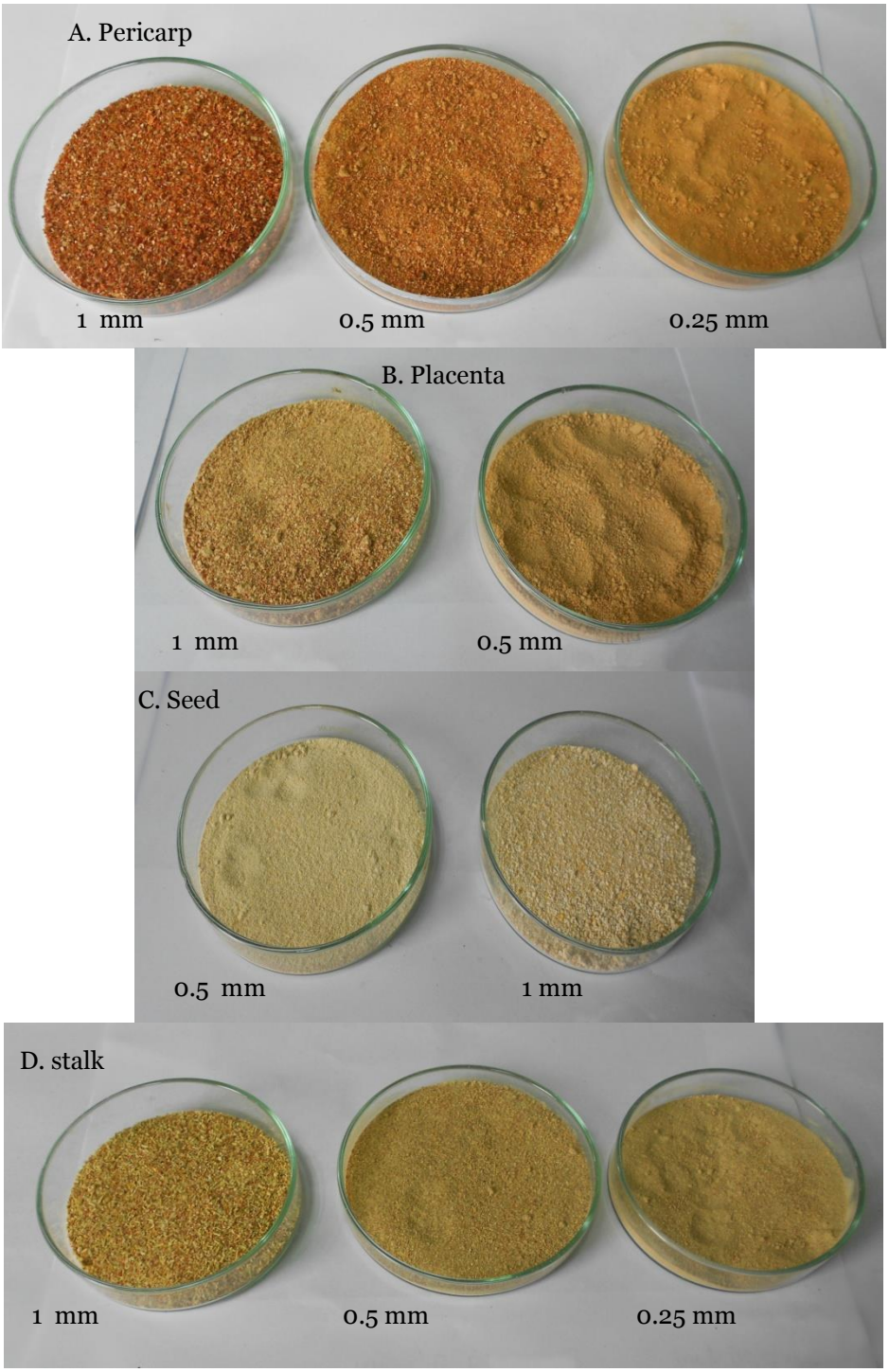


Fig. 5.10. Appearance of A) pericarp, B) placenta, C) seed and D) stalk from red hot pepper with different particle size after extraction with petroleum ether (40-60 ^{o}C).

The values of the colour characteristics determined in the samples after extraction are presented in Table 5.20. The pericarp after the extraction was characterized with the highest value of red color (+a*) compared to placenta, seed and stalk. By increasing the particle size from 0.25 to 1mm, the values for a* for the samples after the extraction increased and ranged from 12.61 to 14.53. The values for a* parameter measured for the placenta with 0.5 and 1.0

mm after extraction were 8.68 and 9.21. In the seed after the extraction, lowest value for a* were determined: 1.70 and 1.99 for seed with 0.5 and 1.0 mm particle size. In the stalk samples, due to the particle size, a* values ranged from 3.26 to 7.34.

Table 5.20. Colorimetric characteristics of pericarp, placenta, seed and stalk of red hot pepper with different particle size after extraction with petroleum ether (40-60 °C).

	Particle size (mm)	Data						
			L*	a*	b*	C	h	BI
Pericarp	0.25	X_{av}	72.98	12.61	28.91	31.55	1.16	62.25
		SD	0.98	0.80	0.46	0.70	0.02	2.48
		RSD (%)	1.35	6.34	1.59	2.23	1.64	3.98
	0.5	X_{av}	70.43	13.12	27.49	30.46	1.13	62.29
		SD	0.61	0.38	0.35	0.33	0.01	1.14
		RSD (%)	0.86	2.89	1.28	1.08	1.16	1.84
	1	X_{av}	61.95	14.54	20.93	25.30	0.96	57.77
		SD	0.72	0.74	0.36	0.44	0.02	1.35
		RSD (%)	1.15	5.06	1.71	1.73	2.04	2.34
Placenta	0.5	X_{av}	73.81	8.68	24.86	26.34	1.23	49.04
		SD	0.89	0.34	0.44	0.47	0.01	1.35
		RSD (%)	1.22	3.89	1.76	1.80	0.89	2.75
	1	X_{av}	74.99	9.21	26.48	28.04	1.24	51.85
		SD	0.49	0.62	0.28	0.43	0.02	1.47
		RSD (%)	0.67	6.72	1.05	1.54	1.52	2.83
Seed	0.5	X_{av}	87.30	1.70	24.24	24.30	1.50	33.22
		STDEV	0.21	0.14	0.30	0.30	0.01	0.59
		RSD (%)	0.24	8.41	1.23	1.24	0.37	1.79
	1	X_{av}	87.11	1.99	22.97	23.04	1.48	31.62
		STDEV	0.46	0.23	0.37	0.37	0.01	0.77
		RSD (%)	0.52	11.72	1.60	1.61	0.64	2.43
Stalk	0.25	X_{av}	73.40	3.26	23.33	23.56	1.43	40.71
		SD	0.30	0.24	0.30	0.30	0.01	0.77
		RSD (%)	0.41	7.30	1.29	1.28	0.69	1.88
	0.5	X_{av}	71.14	3.83	23.44	23.75	1.41	43.10
		SD	0.23	0.10	0.29	0.31	0.01	0.70
		RSD (%)	0.32	2.56	1.25	1.28	0.18	1.63
	1	X_{av}	68.80	7.34	24.28	25.37	1.28	50.56
		SD	0.70	0.47	0.79	0.83	0.02	1.72
		RSD (%)	1.02	6.47	3.25	3.27	1.25	3.39

The colour differences (ΔE) presented in Fig. 5.11. were estimated from the values of the colorimetric parameters before extraction (Table 5.4) and after extraction (Table 5.20).

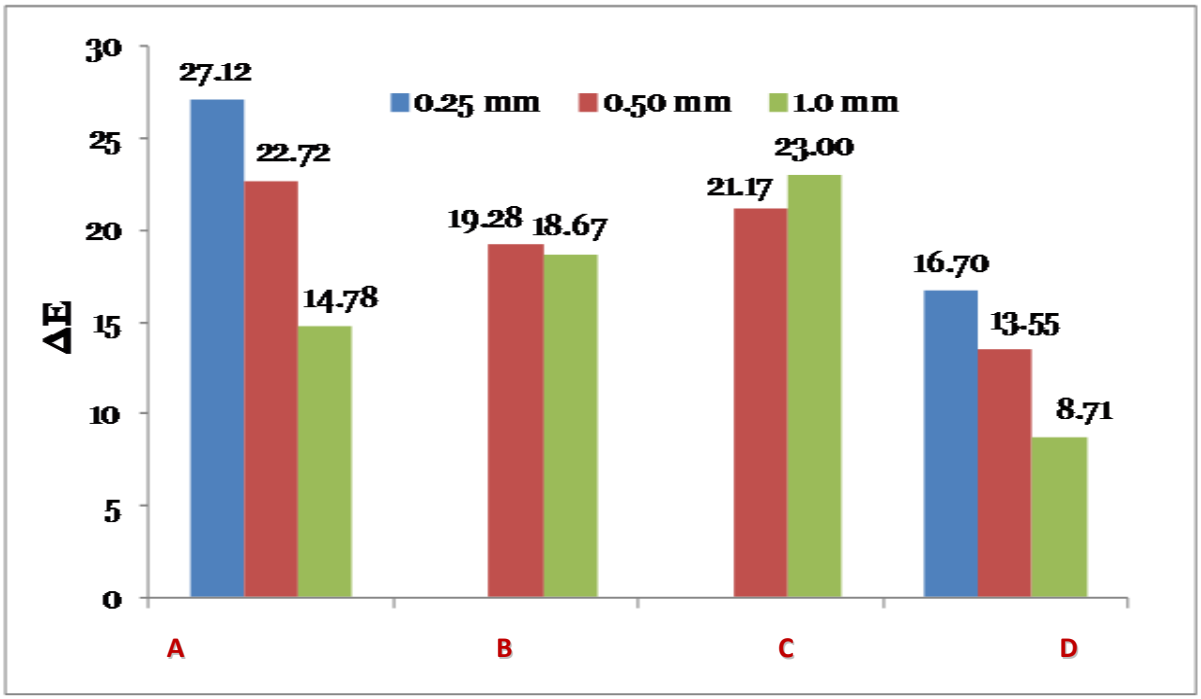


Fig. 5.11. Colour difference (ΔE) of pericarp (A), placenta (B), seed (C), and stalk (D) of red hot pepper with different particle size before and after extraction with petroleum ether (40–60 °C).

Summary for the extraction of pericarp, placenta, seed and stalk. At extraction of pericarp, placenta, seed, and stalk of red hot pepper with petroleum ether (40–60 °C), the bigger particle size enabled to obtain less extract yield. The highest quantities were extracted from seed, while the lowest quantities from stalk. The colour differences increased with decreasing the particle size. The highest colour differences (ΔE = 27.12) were estimated at extraction of pericarp.

5.2.2. Application of sub- and supercritical fluids

5.2.2.1. Extraction with subcritical propane

The extraction with propane was performed at 30 °C temperature and flow rate (2 mL/min) by changing the pressure (20, 50 and 100 bar) and particle size (0.25 mm, 0.5 mm and 1 mm) of sample materials: pericarp, seed, stalk and placenta. The yield of extract was determined. The extracts were analyzed with photochem for the determination of the antioxidative capacity.

Pericarp. In Fig. 5.12. are presented the values of yield of extract obtained at extraction of pericarp with 0.25 mm particle size with subcritical propane at 30°C changing the pressure.

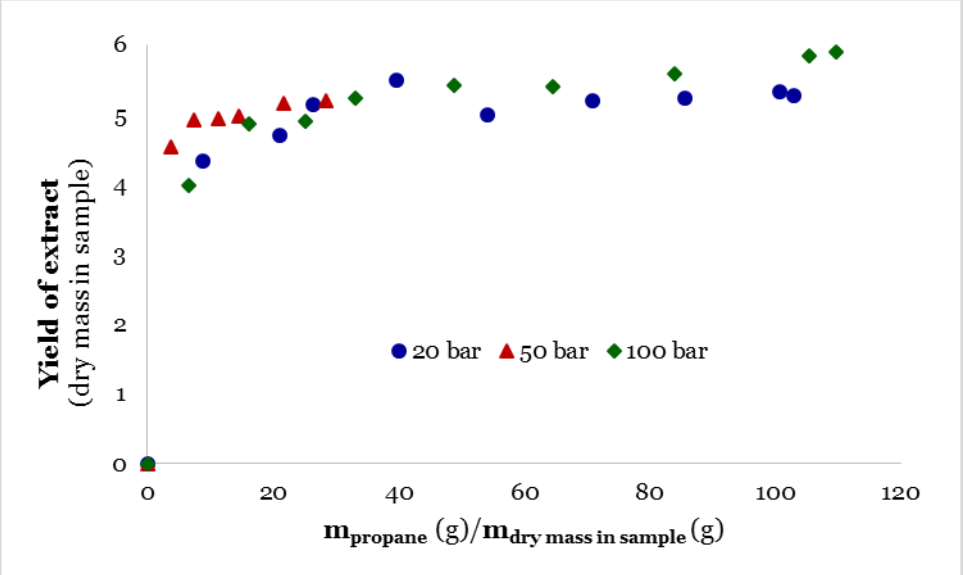
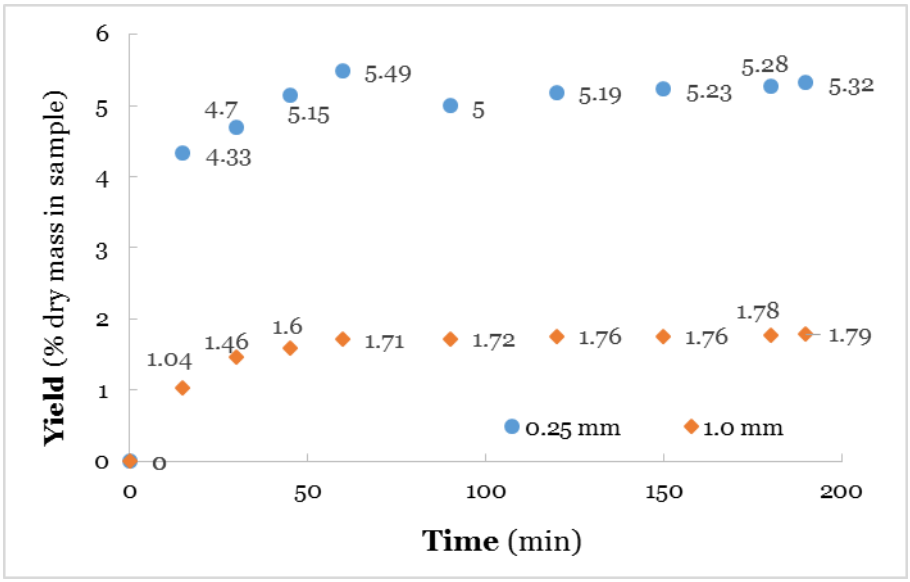
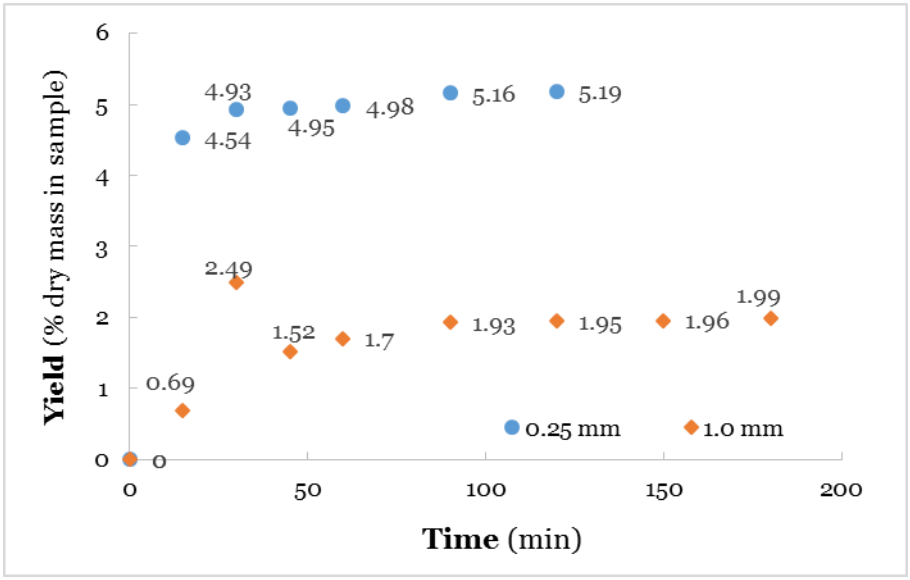


Fig 5.12. Influence of pressure (20, 50 and 100 bar) on the yield of extract of pericarp (0.25 mm particle size) obtained with propane at temperature of 30°C.

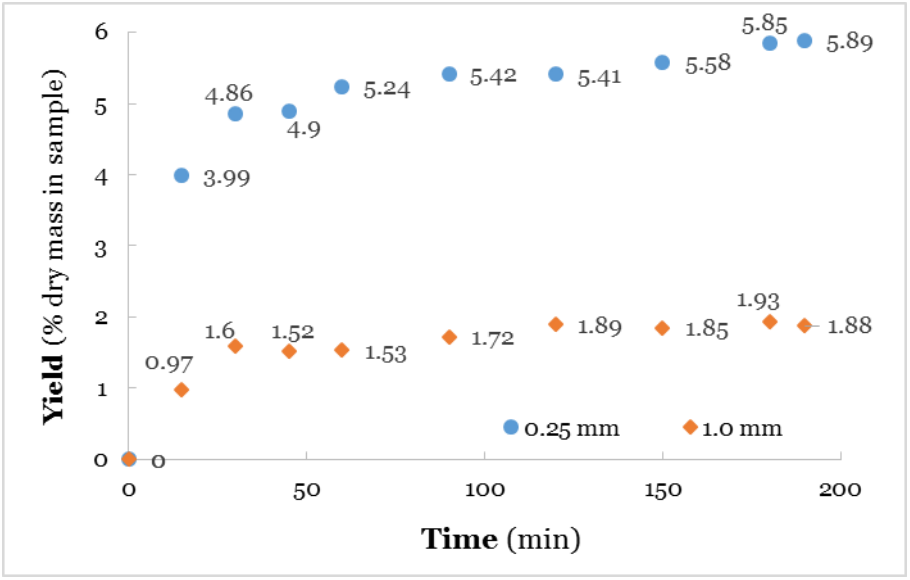
The influence of the pressure (20, 50 and 100 bar) on the yield of the extracts is insignificant. The differences between the yield of extracts obtained from pericarp with 0.25 mm and 1.0 mm particle size by increasing the pressure are insignificant. By degreasing the particle size from 1.0 to 0.25 mm, the highest yield of extracts are obtained. Lipid soluble antioxidant capacity in Trolox equivalents is higher for the extracts obtained from pericarp with 0.25 mm particle size (from 240.19 to 680.32 μg/mg extract)creasing the pressure (20 to 100 bar), antioxidant capacity decreased. Influence of the pressure on the antioxidant capacity of the extract obtained from pericarp with 1.0 mm particle size is insignificant. The difference of the antioxidant capacity are too small (Fig. 5.12 and 5.13).



20 bar



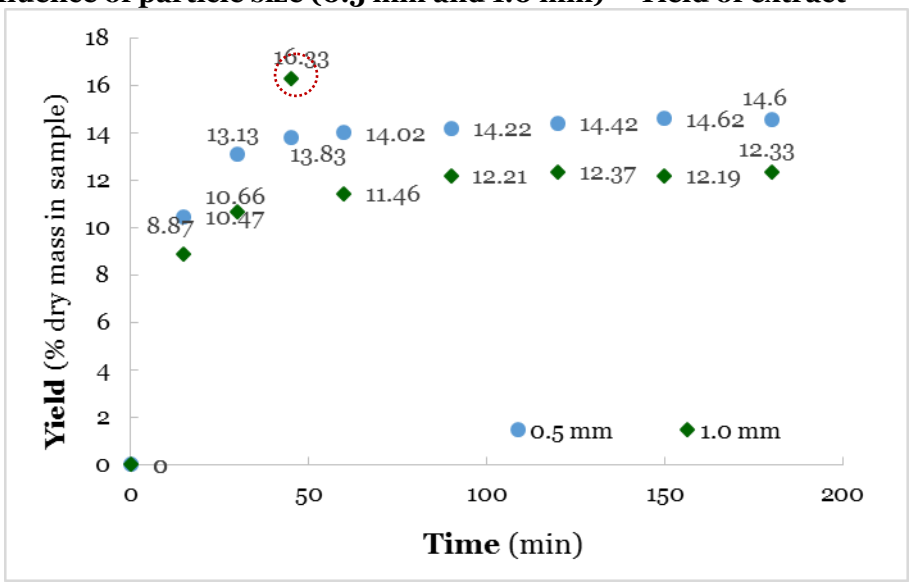
50 bar



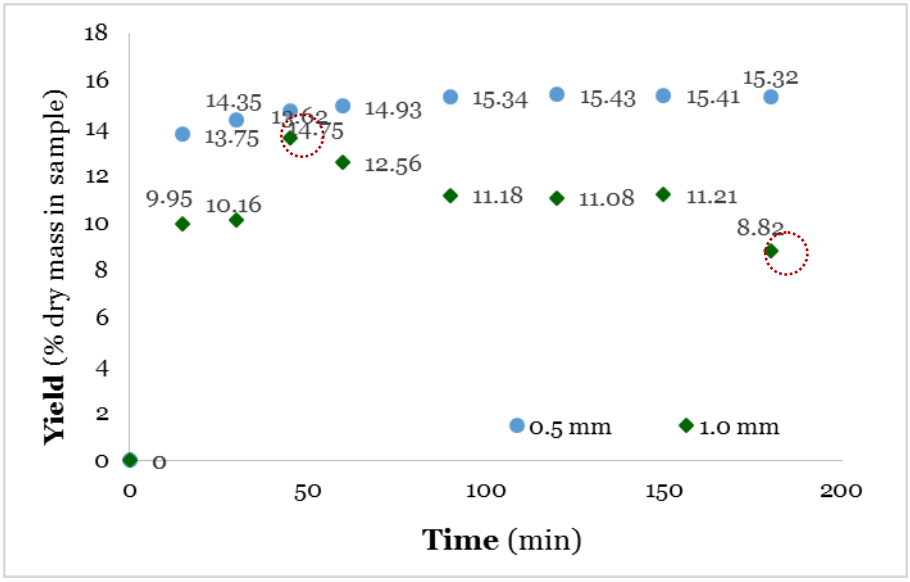
100 bar

Fig. 5.13. Yield of extract (% dry mass in sample) of pericarp (0.25 mm and 1.0 mm particle size) obtained with propane at 30°C, **20 bar**, **50 bar and 100 bar.**

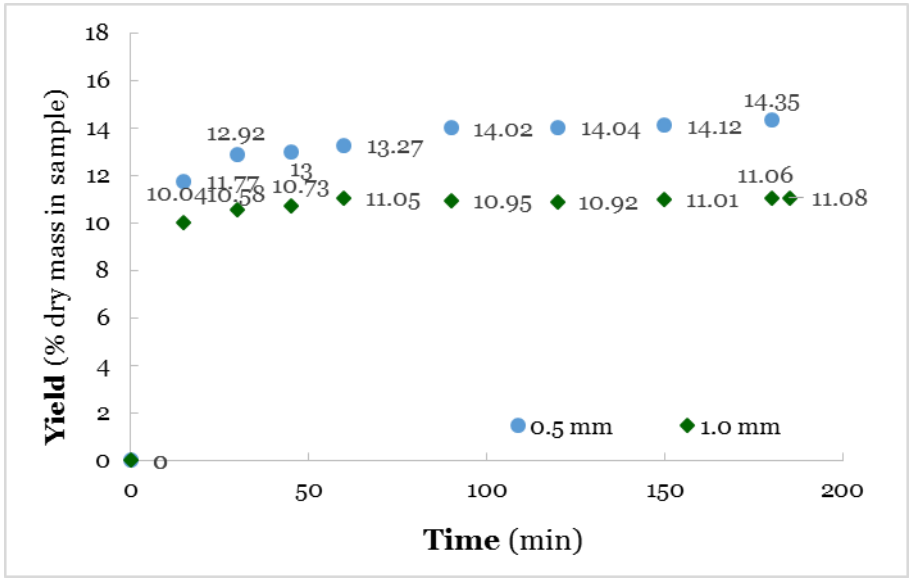
SEED: Influence of particle size (0.5 mm and 1.0 mm) – Yield of extract



A.



B.



C,

Fig. 5.14. Influence of the pressure **(20 bar-A, 50 bar-B and 100 bar-C)** on the yield of extract of seed **(0.5 mm and 1.00 mm particle size)** obtained with propane at 30°C, 180 min, 2 mL/min flow rate (*1 = 185 min at 100 bar).

The differences between the yields of extracts obtained from seed with 0.5 mm and 1.0 mm particle size. By degreasing the particle size from 1.0 to 0.25 mm, the highest yield of extracts are obtained (Fig. 5.14). Lipid soluble antioxidant capacity in Trolox equivalents is higher for the extracts obtained from seed with 1.0 mm particle size (15.83 µg/mg). By increasing the pressure (20 to 100 bar), antioxidant capacity increased. The difference of the antioxidant capacity at pressure of 50 bar between the particle size 0.5 and 1.0 mm of seed are too small.

Placenta. By changing the pressure from 20 to 100 bar, the differences between the yields of extracts obtained from placenta with 0.5 mm particle size are small (Fig. 5.15). Lipid soluble antioxidant capacity (Trolox equivalent) is higher for the extracts obtained from placenta with 0.25 mm particle size (1.83 µg/mg). By increasing the pressure (20 to 100 bar), antioxidant capacity decreased to 1.07 µg/mg.

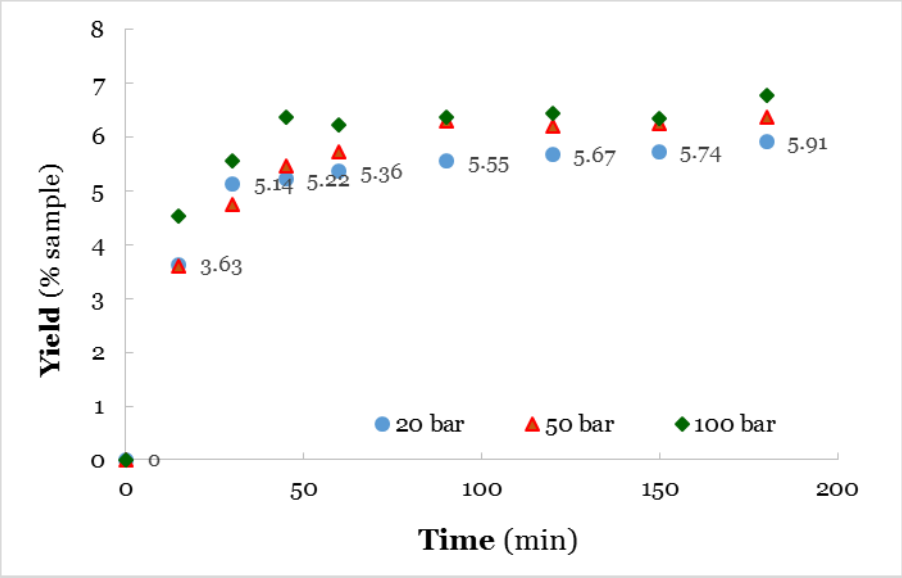
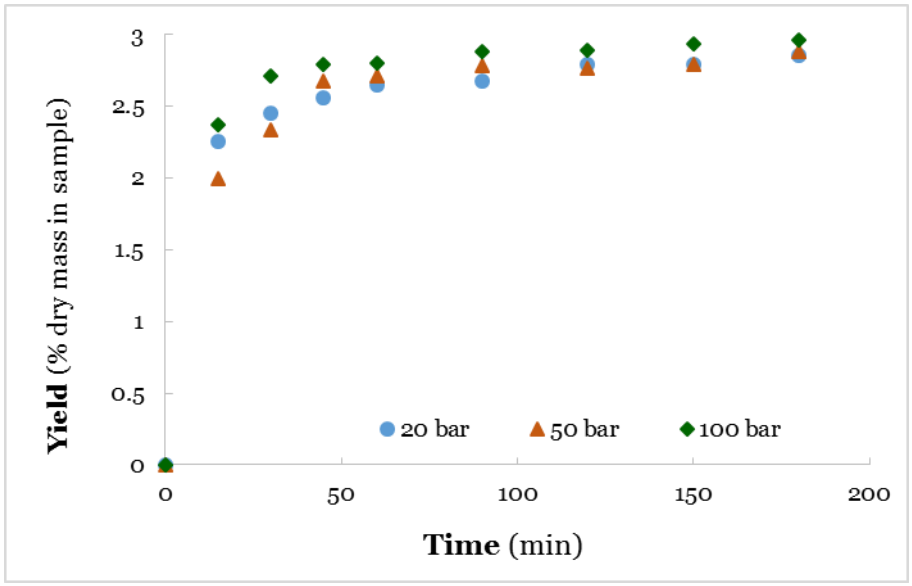
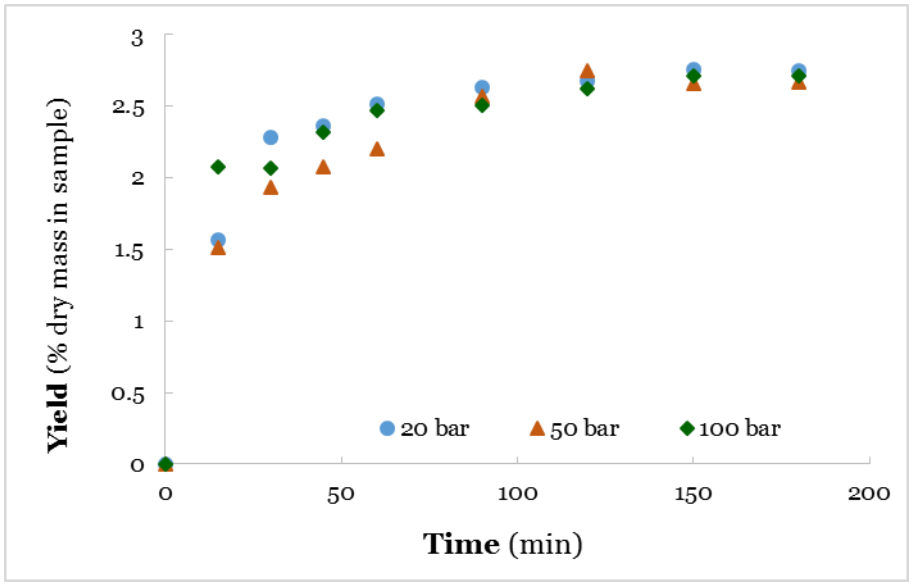


Fig. 5.15. Yield of extract of placenta **(0.5 mm particle size)** obtained with propane at 30°C, **20 bar**, **30 bar and 100 bar**, 180 min, 2 mL/min flow rate.

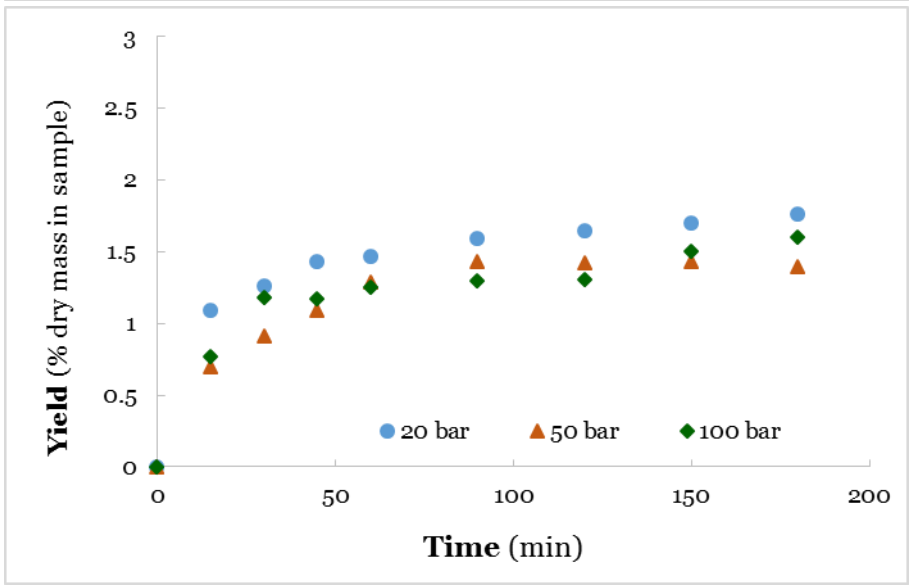
Stalk. The influence of the pressure (20, 50 and 100 bar) on the yield of the extracts from stalk is insignificant. The influence of the particle size on the yield of extract is significant. By degreasing the particle size from 1.0 to 0.25 mm, the highest yield of extracts from stalk are obtained. At 100 bar extraction pressure, the differences in the yield of extract between the particles size of 0.25 and 1.0 mm are higher (Fig. 5.16). The highest lipid soluble antioxidant capacity in Trolox equivalents (2.78 µg/mg extract) shown the extracts obtained from stalk at 50 bar and 1.0 mm particle size.



A.



B.



C.

Fig. 5.16. Influence of the particle size **(0.25 mm-A, 0.5 mm-B and 1.00 mm-C particle size)** on the yield of extract of stalk obtained with propane at 30°C, **20 bar**, **50 bar and 100 bar**, 180 min, 2 mL/min flow rate.

5.2.2.2. Extraction with supercritical carbon dioxide

In the extraction of hot red pepper with supercritical carbon dioxide, on the yield of extract the influence of the particle size of of pericarp (0.25mm, 0.5 and 1.0 mm), placenta (0.25 and 0.25 mm), seed (0.5 and 1.0 mm) and stalk material (0.25mm, 0.5 and 1.0 mm), temperature (40°C) and pressure (400 bar) was researched. The extraction kinetic was followed by taking samples on every 15 minutes in the first hour, and on every hour to constant extract weight achieving.

Pericarp. The yields of extracts obtained from pericarp of hot red pepper at extraction with supercritical carbon dioxide are given in Fig. 5.17. The highest quantity of pericarp extract (5.06%) was obtained from 0.25 mm particle size.

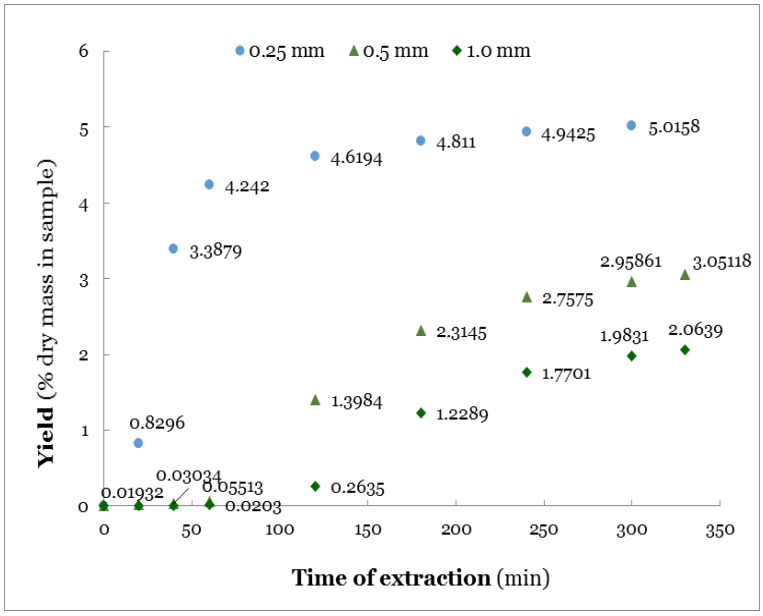


Fig. 5.17. Influence of the particle size **(0.25 mm, 0.5 mm and 1.00 mm particle size)** on the yield of extract of pericarp obtained with CO_2 at **40°C, 400 bar, 330 min.**

Placenta. In Fig. 5.18 are show the values of the yield of extracts obtained at extraction of placenta with supercritical CO_2 at 40°C and pressure of 400 bar. At extraction of placenta with particle size of 0.25 and 0.5 mm, the yield of extracts obtained were 6.2311% and 6.9861. The influence of the particle size of placenta extraction is insignificant, due to the smaller differences obtained in the yields of extracts.

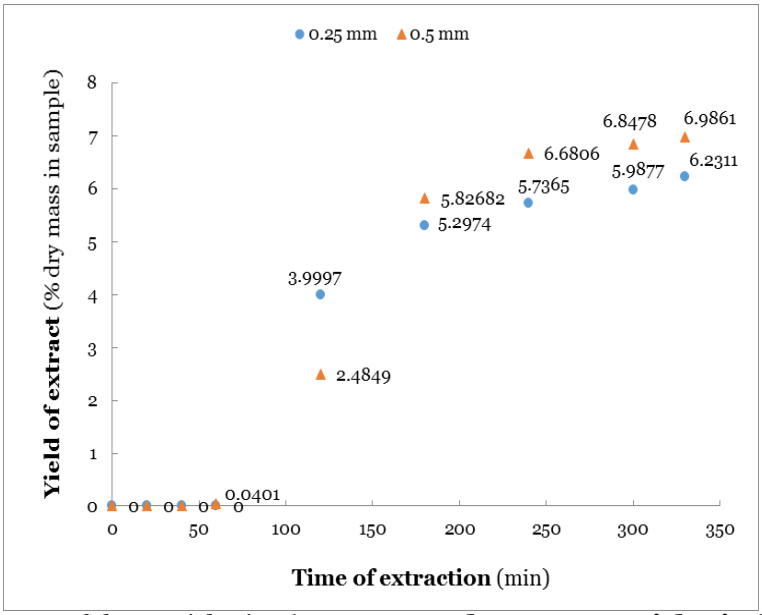


Fig. 5.18. Influence of the particle size **(0.25 mm and 0.5 mm particle size)** on the yield of extract of placenta obtained with CO_2 at **40°C, 400 bar, 330 min.**

Seed and stalk. At SCO_2 of seed with 0.5 mm particle size, at 40°C and 400 bar, the higher yield of extract (19.00%) was obtained compared with the extract obtained from seed with 1.0 mm particle size (9.67%). At extraction with SCO_2 at 40°C and 400 bar, from stalk with 0.5 and 1 mm particle size, the yield of extract of 1.43% and 0.75% was obtained respectively.

In obtained extracts by using Gas Chromatography (GC) method, the content of fatty acids (myristic, palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acid) was determined. The seed extract are characterized with high content of linoleic acid in comparison with the extracts of placenta, pericarp and seed.

The analysis of the aroma compounds in the sample materials (pericarp, placenta, seed and stalk) and obtained extracts was performed by using headspace gas chromatography (HS-GC). In the extract 47 compounds were separated. The concentration of hexyl hexanoate, hexyl isopentanoate, heptyl butanoate, benzyl benzoate, hexyl isobutanoate, hexyl 2- methyl butanoate, beta ionone, alpha ionone, tetradecanoic acid and benzyl benzoate was determined by using analytical standards.

5.3. FORMULATIONS FROM RED HOT PEPPER EXTRACTS

5.3.1. Edible film formulations with seed extract of red hot pepper

Today, due to the negative effect of the plastic packaging materials, the food industry is focusing on alternative packaging films derived from natural biopolymers. The main advantage of the natural biopolymer films produced of raw materials with biological origin against the synthetic films is possibility for their degradation. The main compounds used as a source of edible films used are polysaccharides, proteins, and lipids (Valencia-Chamorro et al., 2011). Namely, polysaccharides as starch and modified starch, pectin, chitin and chitosan, native cellulose and modified cellulose, alginate, and carrageenan are used, while as a source of lipids oils, free fatty acids, bees wax, carnauba wax, paraffin, and terpene resin. Proteins from plant origin (soy proteins, pea and sunflower proteins, wheat gluten, and peanut protein) as well as the proteins from animal origin (whey proteins, collagen and gelatins, casein, egg white protein and fish miofibrilar protein) are reported in the literature as basic material in the preparation of edible films (Šuput et al., 2015). The development of new natural edible films and coatings with antimicrobial compounds and antioxidants for the preservation of food products is a technology challenge for the industry, as well as very attractive research field worldwide (Eça et al., 2014).

The aim of including the capsaicinoids from red hot pepper extracts in the polysaccharide edible film based on sodium alginate was investigated in this doctoral studies. The influence of the quantity of seed extract of red hot pepper obtained by supercritical CO_2 on the edible film characteristics (texture, optical characteristics and antimicrobial activity) was studied.

A. Film thickness and appearance. In Fig. 5.19 the appearance of the formulated and prepared edible films based on sodium alginate with seed extract of red hot pepper as a source of capsaicinoids is presented. The data obtained for the film thickness and appearance are given in Table 5.21.

Fig. 5.19. Appearance of the edible films with different quantity of seed extract of red hot pepper: (a) 10 mg–S_{10}; (b) 20mg–S_{20}; (c) 30 mg–S_{30}; (d) 50 mg–S_{50} and (e) 100 mg–S_{100}.

Table 5.21. Effect of the seed extract of red hot pepper and Na-alginate ratio on the edible film thickness and appearance.

Film code	Na-alginate (%w/v)	Glycerol (%w/v)	Tween 80 (%w/v)	Seed extract and *Na*-alginate (mg/mg)	Film thickness (µm)	Appearance
C	2	0.2	0.2	0	45.02 ± 2.25[a]	Films were transparent, yellowish, elastic, with smooth surface, easy to peel and easy to handle
S_{10}	2	0.2	0.2	1 : 200	42.37 ± 2.89[a]	
S_{20}	2	0.2	0.2	1 : 100	44.06 ± 1.67[a]	
S_{30}	2	0.2	0.2	1 : 67	43.18 ± 2.54[a]	
S_{50}	2	0.2	0.2	1 : 40	41.81 ± 1.97[a]	
S_{100}	2	0.2	0.2	1 : 20	40.60 ± 2.28[a]	

average value ± standard deviation (n=5).

Transparent, slight yellower, homogeneous, thin, flexible and easy to handle films that are completely intact with smooth surface were prepared from 2% Na-alginate solution

and seed extract of red hot pepper obtained with SCO_2. The films were easy to peel from the casting container and also easy for further handling and treatment. The thickness of the edible films is highly important for all physical properties of the films. The influence of the quantity of seed extract is insignificant on the film thickness ($p < 0.05$). The thickness of the Na-alginate film was 45.02 μm, while for the films with seed extract of red hot pepper varied from 40.60 μm to 44.06 μm, respectively, for the film formulated with the ratio of seed extract and *Na*-alginate from 1:20 to 1:100 (Table 5.21).

B. Water vapor permeability and mechanical characteristics. One of the main tasks of the edible films is to prevent the transport of the moisture between the food and environmental surrounding. With that aim, is very important to determine *water vapor permeability* of the Na-alginate films modified with the seed extracts of red hot pepper. The alginates films were characterized with the water vapor permeability from 1.98 to 2.45 moL·m/m²·s·Pa. Increasing the seed extract of red hot pepper from 20 to 100 mg, slight increase in water vapor permeability was determined. The differences are statistical insignificant ($p < 0.05$) and quantity of seed extract added in quantity to 100 mg was ineffective on the film barrier characteristics (Table 5.22). The values determined for *mechanical properties* (puncture tension, deformation and puncture force) of the alginate films modified with seed extract are presented in Table 5.22. The quantity of seed extract of 30 mg is not statistical significant ($p < 0.05$) for the puncture tension and puncture of the films, while show the significant effect on the percentage of sample deformation. The high amount of seed extract (50 and 100 mg) enabled the increasing of the puncture tension and force for two times (278 kPa and 27.82 N, respectively), also, the deformation is increased.

Table 5.22. Water vapor permeability and mechanical characteristics of edible films with different ration of seed extract and *Na*-alginate.

Film code	Water vapor permeability (moL·m/m²·s·Pa) · 10^{-11}	Mechanical characteristics		
		Puncture tension (kPa)	Sample deformation (%)	Puncture force (N)
C	2.04	110.22 ± 10.54^{a}	2.90 ± 0.30^{a}	8.12
S_{10}	1.98	144.98 ± 9.83^{a}	3.84 ± 0.08^{b}	11.22
S_{20}	2.39	102.65 ± 12.04^{a}	3.52 ± 0.17^{b}	11.01
S_{30}	2.29	108.97 ± 11.78^{a}	3.37 ± 0.39^{b}	11.95
S_{50}	2.45	223.39 ± 14.89^{b}	4.87 ± 0.25^{c}	23.14
S_{100}	2.31	278.23 ± 13.56^{a}	6.16 ± 0.73^{d}	27.82

average value ± standard deviation (n=5); ^{a,b,c} Values with different superscripts within a row are significantly different (Tukey test, $p < 0.05$).

C. Optical properties. The optical properties of the edible films are very important attributes because they may directly influence the consumer's acceptability of the product. The color parameters (lightness, L; color saturation, C and hue, h) and whiteness index for all film formulations are presented in Table 5.23. The lightness (L*) of the film

samples of Na-alginate films were varied from 75.24 to 84.89. The addition of the seed extract of the red hot pepper, glycerol and Tween 80 to the Na-alginate solution resulted in films with slight yellow appearance, the b* values ranged from 4.38 to 5.90. The whiteness index of the control edible films and films with quantity of seed extract of 50 and 100 mg was 82.49, 83.91 and 83.68.

Tabela 5.23. Colour parameters and whiteness index of edible films with different ratio of seed extract and *Na*-alginate.

Film code		Color parameter					Whiteness index - WI
		L*	a*	b*	C	h	
C	1	83.29^{a}	-0.32^{a}	5.23^{a}	2.10^{a}	87.63^{a}	82.49^{a}
	2	1.13	0.02	0.10	0.03	0.12	1.01
S_{10}	1	79.79^{ab}	-0.26^{b}	5.90^{ab}	2.66^{ab}	87.95^{a}	78.95^{a}
	2	2.99	0.03	0.77	0.69	0.22	3.07
S_{20}	1	75.24^{c}	-0.23^{c}	6.51^{b}	3.23^{b}	89.21^{a}	75.68^{a}
	2	1.60	0.02	0.72	0.73	0.76	2.44
S_{30}	1	83.91^{a}	-0.28^{ab}	5.47^{ab}	2.64^{ab}	87.08^{ab}	80.53^{a}
	2	0.76	0.03	0.03	0.17	0.26	4.33
S_{50}	1	84.53^{a}	-0.23^{ab}	4.38^{c}	0.96^{c}	86.32^{a}	83.91^{a}
	2	2.23	0.02	0.29	0.69	0.23	2.08
S_{100}	1	84.89^{a}	-0.28^{ab}	4.55^{c}	0.10^{c}	86.59^{ab}	83.68^{a}
	2	0.71	0.01	0.03	0.01	0.02	0.67

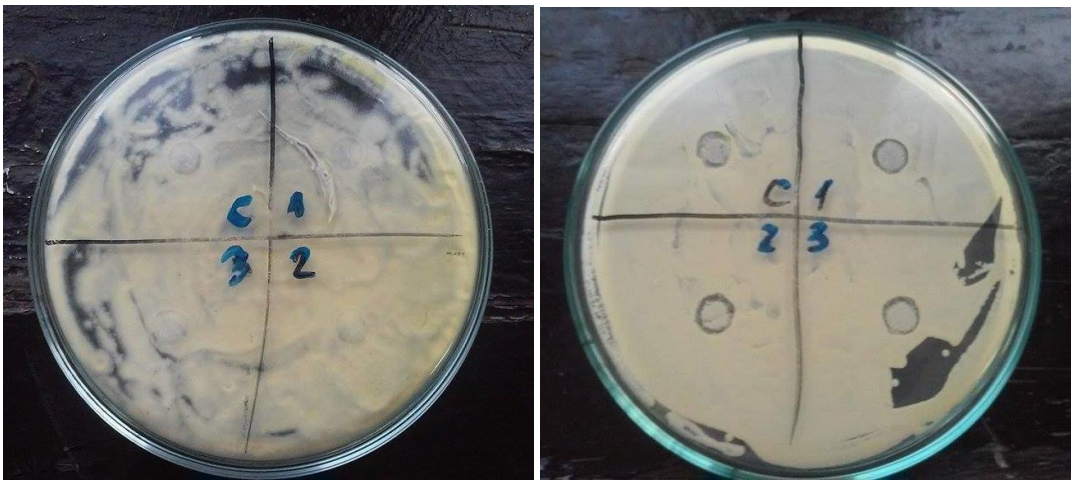
1 – average value (n=5); 2 – standard deviation; *a,b,c* Values with different superscripts within a row are significantly different (Tukey test, $p < 0.05$).

The light transmission of the film in a wavelength range from 200 to 800 nm was 8.7–40 % in the range of visible wavelength (400–800 nm), while the transparency varied from 11 to 18% (Table 5.24). The seed extract of red hot pepper not provoke turbidity of the films and losses in transparency, which is very important for the further application of films.

Table 5.24. Light transmittance (%) and transparency values of edible films with different ratio of seed extract and *Na*-alginate.

Film code	Wavelength (nm)				Transparency (%)
	200	400	600	800	
C	0	19.4	31.0	40.0	11.29
S_{10}	0	8.7	17.1	25.4	18.13
S_{20}	0	9.2	17.7	26.1	17.06
S_{30}	0	13.4	24.5	33.6	14.14
S_{50}	0	20.3	30.0	40.0	12.50
S_{100}	0	13.8	23.2	32.6	15.66

D. Antimicrobial activity. The antimicrobial activity of the edible films was tested against the Penicillum mould and yeast that were isolated from the soft white brined cheese. The edible films of Na-alginate with seed extract of red hot pepper was not effective against Penicillum mould (Fig. 5.20a). Small inhibition zones were determined of edible films against the yeast (Fig. 5.20b).



(a) (b)

Fig. 5.20. Activity of the edible films of Na-alginate modified with seed extract of red hot pepper against *Penicillum* (a) and yeast (b).

5.3.3. Nanoemulsion formulations of seed extract of red hot pepper

An emulsion is a suspension of two immiscible liquids, *water* and *oil*, in which small oil droplets is dispersed in an aqueous phase or vice versa. The relatively simple formation and processing methods allow the emulsions to be widely utilized as colloidal delivery system of various kind of nutraceuticals and pharmaceutical compounds. Emulsions are a dispersion of an immiscible liquid into another stabilized by the presence of a third compound *surfactant*. Emulsion systems are classified as *oil-in-water (O/W) emulsion* if small oil droplets are dispersed in an aqueous phase, and *water-in-oil (W/O) emulsion* consist of water droplets dispersed in an oil phase. The emulsions are generally divided into three categories based on their stability and droplet size: microemulsions, nanoemulsions and macroemulsions. *Microemulsions* are systems with droplet size 1-200 nm and they are thermodynamically stable which means that they will form spontaneously. Systems labeled as *naemulsions* and *macroemulsion* consisted of droples with sizes in the range of 10-400 nm (nanoemulsion) and 0.5-50 μm (macroemulsion) are kinetically stable and over time they will phase separate to yield a lower energy state. Nanoemulsion is considered to be kinetically stable as it exhibits random Brownian motion that is sufficient in overcoming gravitational separation and thus prevents emulsion droplets from creaming and sedimentating as well as re-coalescence and flocculation. By choosing the correct emulsifiers, it is possible to have a stable system for a long period of time. Usually, using a mixture of surfactants with various values of *Hydrophile-Lipophile Balance* (HLB) to match the required HLB of the oil provides

better coverage of the oil surface than using a single surfactant (Mason et al., 2006; McClements and Rao, 2011). Nano-emulsions can be transparent or translucent (size range 50-200 nm) or "milky" (up to 500 nm). The longterm physical stability of nano-emulsions (with no apparent flocculation or coalescence) makes them unique, and they are sometimes referred to as "approaching thermodynamic stability" (Izquierdo et al., 2001; De Azevedo Ribeiro, 2015). Nanoemulsion can be fabricated by *low energy* or *high energy emulsification* methods. In the *low-energy* method occurs spontaneous formation of tiny oil droplets within mixed oil-water-emulsifier systems when the solution or environmental conditions are altered, e.g., phas einversion and spontaneous-emulsification methods. *High energy* emulsification requires the input of energy to generate disruptive forces that mechanically breakup large oil droplets to form fine droplets that are suspended within the aqueous phase. One of the advantages of using this method is that nanoemulsion can be easily achieved by using a lower amount of emulsifier. Also, it offers flexible control over droplet size and droplet size distribution and a greater improvement in stability, as well as being potentially applicable for large-scale industrial production. Energy-intensive technologies such as ultrasonication, high pressure homogenization and microfluidization are commonly used for the preparation of nanoemulsions (Salvia-Trujillo et al., 2016).

Currently, nano-scale delivery systems, has become a promising approach for incorporating various type of compounds, *nutraceuticals* and *phytochemicals*, with aim improving the stability, as well as the solubility of the compounds (FAO/WHO, 2010; Paul and Dewangan, 2016). The formation of the new carries for topical delivery of pure capsaicin is reported by the Tavano et al. (2011).

The application of the nanoemulsion methods in the formulation of emulsion systems from extracts of red hot pepper was studied in this the doctoral thesis. Nanoemulsion formulations containing active ingredients of seed red hot peper extract obtained by supercritical CO_2 and non-ionic surfactant (Tween 80, Tween 20 and Span 80) were prepared by spontaneous emulsification method and high energy method.

A. Spontaneous emulsification method. As is shown in Fig. 5.21, by application of the spontaneous method, emulsion systems with "milky" colored appearance were obtained.

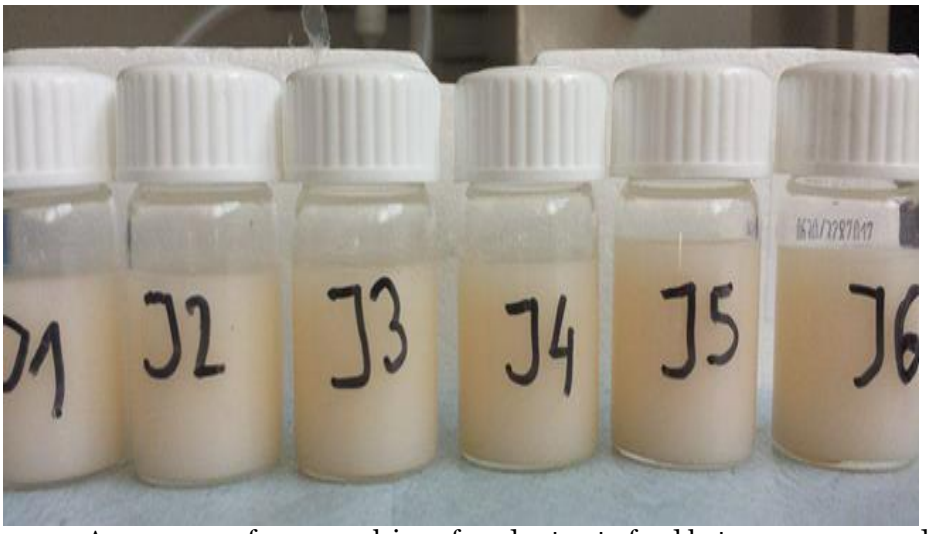


Fig. 5.21. Appearance of nanoemulsion of seed extract of red hot pepper prepared by spontaneous emulsification method.

The values measured for the droplet size, polydispersity Index (PDI) and ζ-potential of prepared nanoemulsion with different ratio of seed extract (SE) of red hot pepper and mix of surfactants Tween 80, Tween 20 and Span 80 are presented in Table 5.25.

In the nanoemulsion obtained by *mix of surfactants Tween 80* and *Span 80* (SmixT80), droplet sizes were 263.9 nm, 277.8 nm and 263.2, respectively, for ratio of SE and SmixT80 of 1:1, 1.5:1 and 2:1. The PDI increased from 0.151 to 0.21 by increased of the SE and Smix T80. Very small values of the ζ-potential were determined. The ζ-potential varied from -0.84 to -5.08 mV. As shown in Table 5.25, the results demonstrated that average droplet size, PDI and ζ-potential remain fairly unchanged throughout the storage period of 10 days at 4 °C, while at 25 °C slightly increasing in droplet size was determined.

The nanoemulsion formulated by SE and *mix of surfactants Tween 20* and *Span 80* (SmixT20) are with higher droplet sizes (around 320 nm) compared to SmixT80. The droplet sizes increased to 418 nm for nanoemulsion of SE and SmixT20 that was storage 10 days at at 25 °C (Table 5.25).

Table 5.25. Z-average diameter, PDI and ζ-potential of red hot pepper seed extract (SE) nanoemulsions prepared by spontaneous emulsification method at different ratio of SE and surfactant mix.

Parameter	Storage conditions		SE : SmixT80[1]			SE : SmixT20[2]		
	Time (day)	Temperature (°C)	**1 : 1**	**1.5 : 1**	**2 : 1**	**1 : 1**	**1.5 : 1**	**2 : 1**
Z-average diameter (nm)	1	4	263.9±4.37	277.8±0.99	263.2±4.16	317.2±5.08	269.1±1.70	289.0±4.46
		25	263.0±1.21	279.8±0.71	262.0±2.26	325.1±2.54	268.2±1.62	288.0±6.48
	5	4	274.5±4.07	285±0.85	273±1.67	310.7±4.22	264.9±2.47	281.9±3.37
		25	261.9±1.54	294.6±1.36	278.6±11.38	306±2.31	268.2±2.05	282.5±2.91
	10	4	266.2±4.55	286.3±2.16	288.9±3.12	359±17.17	265.6±4.37	417.9±8.42
		25	325.8±23.78	282.5±6.77	300.6±6.46	323.9±0.71	264.4±4.01	345.6±9.66
Polydispersity Index– PDI	1	4	0.151±0.008	0.199±0.019	0.219±0.006	0.204±0.017	0.182±0.014	0.206±0.018
		25	0.144±0.009	0.179±0.010	0.188±0.035	0.225±0.011	0.184±0.013	0.217±0.023
	5	4	0.207±0.006	0.185±0.015	0.235±0.003	0.185±0.037	0.151±0.024	0.214±0.008
		25	0.135±0.038	0.244±0.006	0.251±0.022	0.184±0.010	0.162±0.023	0.201±0.013
	10	4	0.176±0.035	0.185±0.013	0.307±0.009	0.286±0.037	0.148±0.004	0.396±8.422
		25	0.308±0.037	0.209±0.020	0.306±0.044	0.228±0.005	0.155±0.003	0.356±0.063
ζ-potential (mV)	1	4	-2.11±0.650	-1.99±0.722	-3.56±0.695	-1.72±0.14	-0.775±0.29	-0.558±0.244
		25	-1.078±0.156	-1.07±0.611	-5.08±0.605	-2.62±0.436	-1.01±0.188	-1.45±0.335
	5	4	-2.79±0.252	-1.56±1.530	-3.08±0.055	0.377±2.16	1.89±2.33	-2.33±1.61
		25	-1.88±2.240	-1.85±0.751	-3.8±0.516	-0.847±3.06	-0.096±2.02	-3.98±1.45
	10	4	-6.12±4.66	-0.151±0.644	-2.13±0.52	1.34±6.27	-1.22±1.32	- 3.11±1.82
		25	-2.16±3.47	-0.275±0.0933	-3.75±0.468	-5.13±4.097	-1.63±1.29	- 1.93±1.49

[1]SmixT80–(Tween 80:Span80 = 1.5:1); [2]SmixT20–(Tween 20:Span80 = 1.5:1).

The transmission electron micrographs of the nanoemulsions prepared by SE and SmixT80, and SE and SmixT80 in ratio of 1.5:1 is presented in Fig. 5.22. Based on TEMs in nanoemulsion of SE and SmixT80 with HLB of 10.7 (Fig. 22a and b) spherical in shape and uniform in size oil droplets were observed. In the nanemulsion formulation of SE and SmixT20 (HLB=11.32) the bigger droplet size were observed (Fig. 22 c and d).

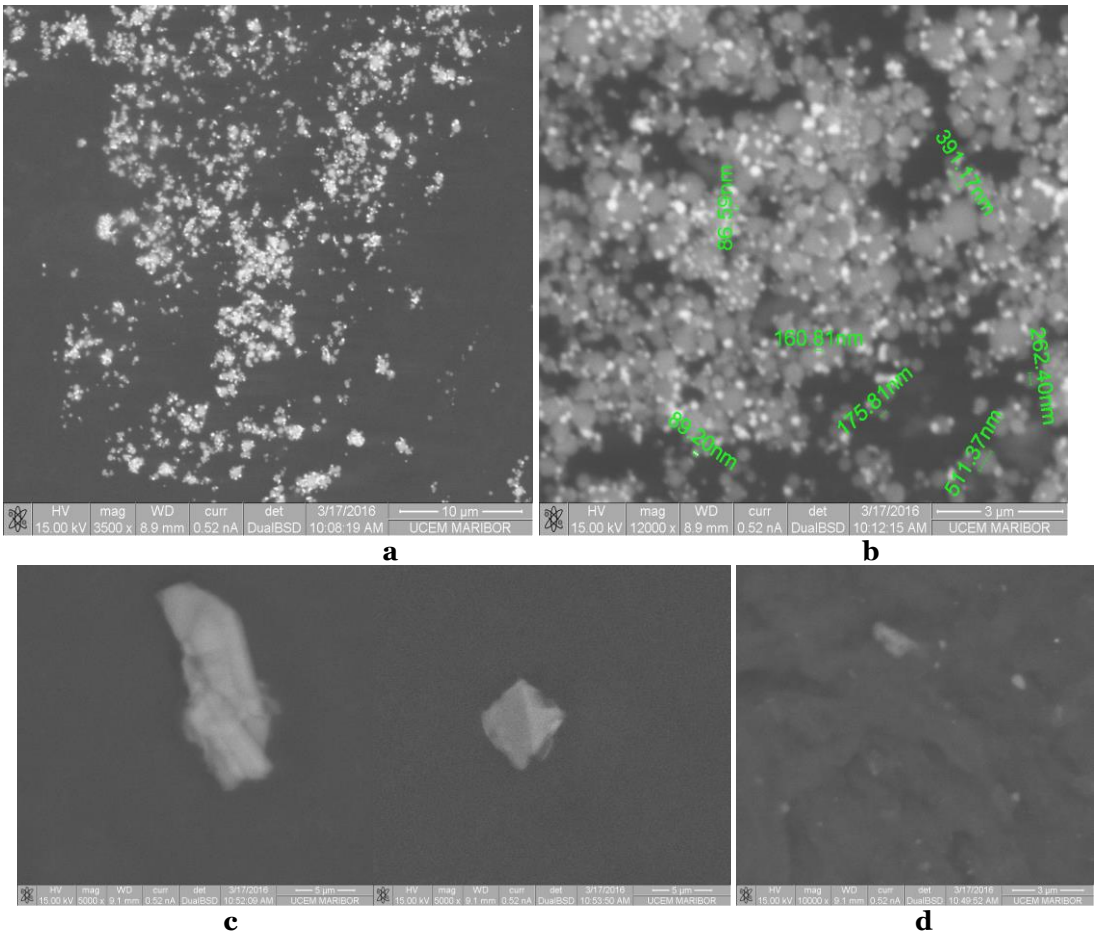


Fig. 5.22. Transmission electron micrographs of red hot pepper seed extract nanoemulsions obtained by spontaneous emulsification method:
(a and b) SE and SmixT80 in ratio of 1.5:1 (magnification 3 500 x and 12 000x),
(c and d) SE and SmixT20 in ratio of 1.5:1 (magnification 5 000 x and 10 000 x).

B. High energy emulsification method. In Fig. 5.23, by is presented the appearance of the nanoemulsions prepared by high energy emulsification method.

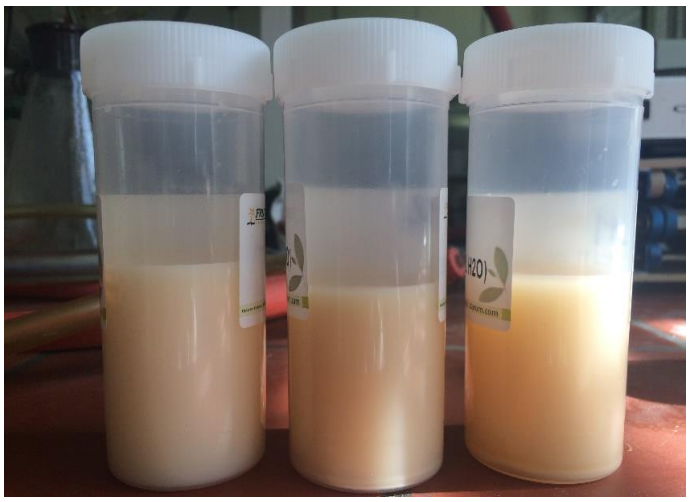


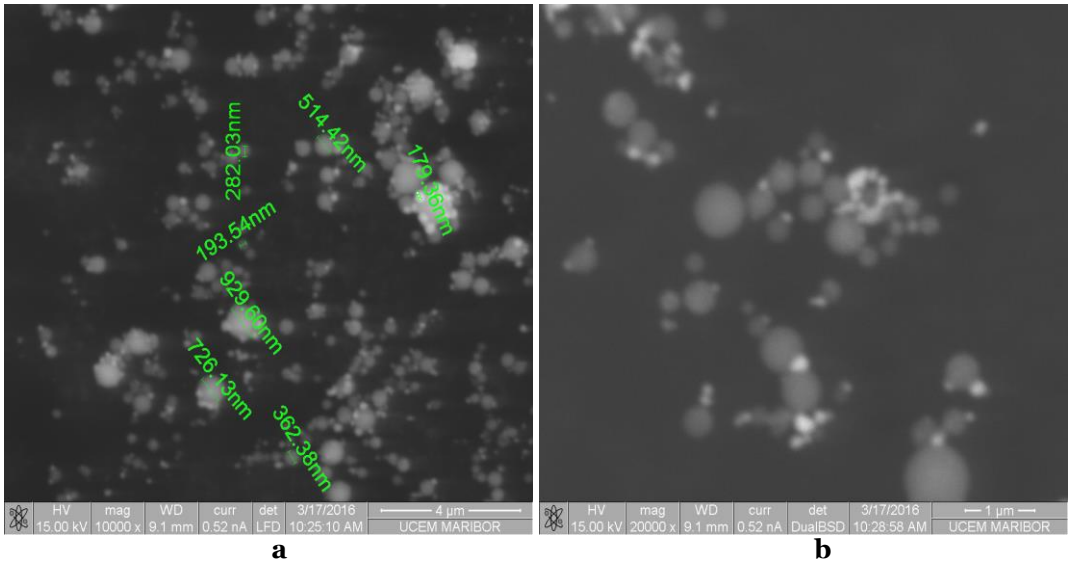
Fig. 5.23. Appearance of nanoemulsion of seed extract of red hot pepper prepared by high energy method.

In Table 5.26 are given the values for Z-average diameter of droplets, PDI and ζ-potential of the nanoemulsion of red hot pepper seed extract (SE) prepared by high energy method where the in the formulation polyethylene glycol was used as SE solvent, and different quantities of gelatin polymer and phospholipid lecithin were used, also. Emulsification process was performed in two types of homogenizer: type APV – 2000 and Polytron PT1200. The influence of the homogenizer and conditions of mixing and emulsification was not significant, due to the differences of droplet size, PDI and zeta potential obtained by application of the two homogenizer types. The smallest droplets (206.7 and 285.0 nm), and PDI (0.218 and 0.223 nm), respectively for homogenizer type APV – 2000 and PT1200) were obtained by using SE and gelatin. In Fig 5.24 is given TEM of the nanoemuslion of SE with gelatin in ratio of 1:1. In the formulation obtained by introduction of lecithin, the higher values for droplet size, PDI and zeta potential were determined. The highest values of droplet size (693 nm) and PDI (0.94) were obtained for the formulation of SE, gelatin, lecithin in ratio of 1.0:0.6:0.5, prepared with homogenizer PT1200. For the formulation of SE, gelatin, lecithin in ratio of 1.0:0.6:1, by application of PT1200 were obtained droplet size of 312.9 nm, 0.786 PDI and the highest value zeta potential (-29.8 mV) compared to other formations (Table 5.26).

Table 5.26. Z-average diameter, PDI and ζ-potential of red hot pepper seed extract (SE) nanoemulsions prepared by high energy method with different amount of gelatin and phospholipid lecithin.

Characteristic	Type of homogenizer	Nanoemulsion formulation		
		[1]SE : gelatin	[2]SE : gelatin + lecithin	[3]SE : gelatin + lecithin
Z-average diameter (nm)	*1	285.0±2.05	603.1±21.06	435.2±26.49
	*2	206.7±1.62	513.6±9.45	312.9±4.56
Polydispersity Index– PDI	*1	0.218±0.01	0.94±0.06	0.705±0.001
	*2	0.223±0.013	0.567±0.049	0.786±0.016
ζ-potential (mV)	*1	0.587±0.223	-21.3±0.751	-19.5±0.702
	*2	4.7±0.176	-23.3±1.470	-29.8±0.651

*[1]high pressure homogenizer type APV – 2000; *[1]high pleasure homogenizer Polytron PT1200; [1]SE : gelatin = 1:1; [2]SE : gelatin : lecithin = 1 : 0.6 : 0.5; [3]SE : gelatin : lecithin = 1 : 0.6 : 1.



a **b**

Fig. 5.24. Transmission electron micrographs of red hot pepper seed extract nanoemulsion obtained by high energy method (a and b) [1]SE : gelatin = 1:1 (magnification 10 000x and 20 000x), (c and d) SE and SmixT20 in ratio of 1.5:1 (magnification 5 000 x and 10 000 x).

5.3.3. Biopesticide formulations of extracts of red hot pepper

Despite advances in integrated pest management, and frequent use of insecticides, there is a need to shift emphasis on biological control agents and phytoproducts especially plant extracts and oleoresins (Gašić and Tanović, 2013).

Fruits of red hot pepper (*Capsicum annuum* L., *Solanaceae*) are valuable source of secondary metabolites, alkaloids like capsaicinoids and steroidal glycosides, well known due to their biological activity (Materska and Perucka, 2005). The highest amount of capsaicininoids is found in the placenta of the fruit, while the pericarp and seed the content

of capsaicinoids is lower (Pandhair and Sharma, 2008). The possibility of application of the extracts of red hot pepper in contoll the pests in agricultute crops as alterantive of chemical pesticide was evaluated. The effects of the crude extracts from the pericarp, stalks, placenta and seeds of red hot pepper (*Capsicum annuum* L., ssp. *microcarpum longum conoides*, convar. Horgoshka) against green peach aphid pest (*Myzus persicae* Sulz) on tobacco plants were studied. Green peach aphid has been a significant pest of tobacco in Republic of Macedonia for many years, because of its capacity to transmit viruses (Krsteska et al., 2007). One of the most serious problems of the tobacco production concerns the green peach aphid, (*Myzus persicae* Sulz.) insect which transmit viruses and damage tobacco by removing plant juices and contaminating leaves with cast skins and honeydew (Fig. 5.25).

Fig. 5.25. Infestation of tobacco with green peach aphid pest (*Myzus persicae* Sulz.).

Laboratory experiment. In Table 5.27 and Fig. 5.26, the effectiveness of biopesticide preparations from extracts of red hot pepper and the commercial insecticide Confidor SL 200 in the control of green tobacco aphid determined in laboratory conditions is presented.

Table 5.27. Efficiency of biopesticide from red hot pepper extracts and insecticide Confidor SL 200 in control of tobacco green peach aphid pest-*laboratory conditions*.

Formulation		Pests on tobacco leaves before treatment (number)*	Pests on tobacco leaves 24 hours after treatment (number)*	
			living	*dead*
Extract of	pericarp	303	57	246
	placenta	258	10	248
	seed	271	201	70
	stalk	289	162	127
Insecticide	Confidor Sl 200	292	0	292

* number of leaves = 4.

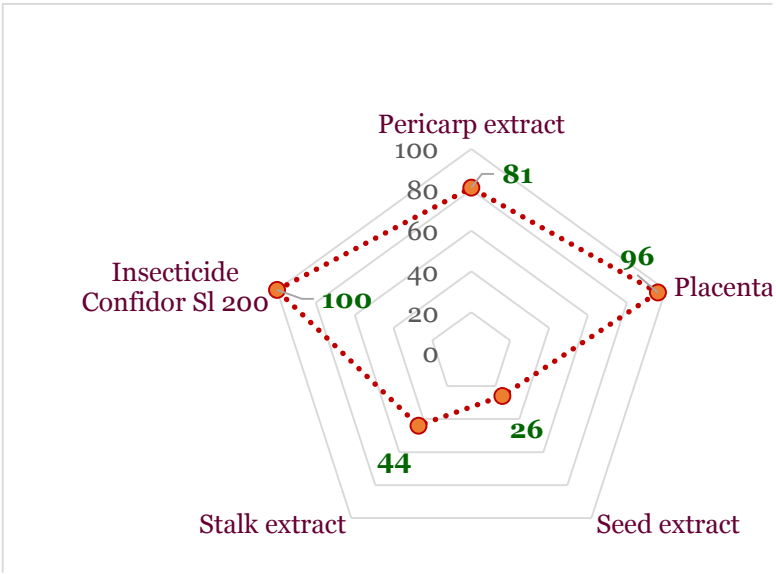


Fig. 5.26. Efficiency according to Aboot (%) of extracts of red hot pepper and insecticide Confidor SL 200 in control of aphids on tobacco leaves.

In laboratory trial against aphids compared with the imidacloprid effect (100%), the followed efficacies of the red hot pepper extracts were determined: placenta extract (96%), pericarp extract (81%), stalk extract (44%) and seed extract (26%), Fig. 5.26.

Experiment in greenhouse. The highest mortality of aphid population in greenhouse was estimated in the treatment with the imidacloprid (99%). Satisfactory results in aphid control were obtained by application of placenta extract (67%) and pericarp extract (48%). The lowest aphid mortality was determined with the extracts of stalks (28%) and seed (11%). The increasing of the aphid population mortality was insignificant 48 hours after treatment with extracts of red hot pepper fruits Fig. 5.27.

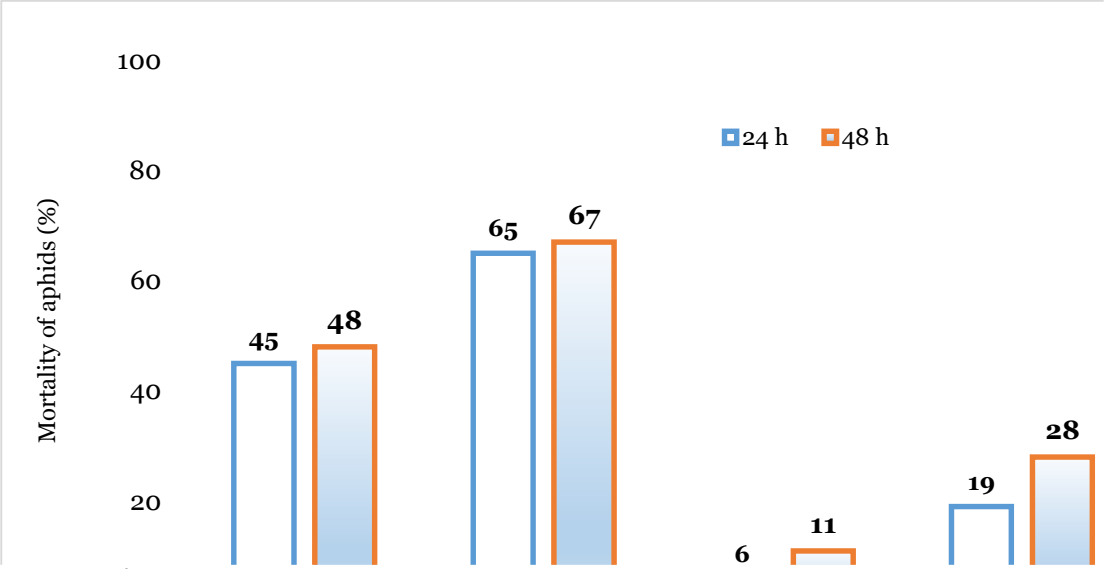


Fig. 5.27. Mortality of aphids (%) after treatment with pepper extracts and imidacloprid efficiency according to Aboot (%) of extracts of red hot pepper and insecticide Confidor SL 200 in control of aphids on tobacco leaves.

Summary for biopesticides. The pepper extracts can be good insecticidal options for Integrated Pest Management Program in the control of green peach aphid (*Myzus persicae* Sulz) in tobacco. They are selective and showed a less negative impact on the ecosystem. The placenta extract shown significantly highest efficacy against green peach aphid on tobacco plants, followed by pericarp extract.

6. CONCLUSIONS

According to the results obtained at characterization of the red hot pepper, and extraction of oleoresins from pericarp, placenta, seed and stalk, and formulation of extracts in edible films, nanoemulsions and biopesticide, the following conclusions acquired.

Plant material characterization. The fruits of hot pepper variety *Capsicum annuum* L., ssp. *microcarpum longum conoides*, convar. Horgoshka, cultivated in the Republic of Macedonia are characterized by high quantities of nutritional and bioactive constituents. The pericarp, as the edible part of fruit, is a good source of carotenoids, dietary fiber and reductive sugars. The seed and placenta are characterized by a valuable content of proteins and micro- and macroelements. In the placenta, in comparison to the pericarp and seed, over 60% of the total quantity of capsaicinoids, capsaicin and dihydrocapsaicin was found. The highest qualitative ratio of capsaicin and dihydrocapsaicin was found in the pericarp. The high quantities of capsaicinoids determined in the placenta and seed of pepper fruits emphasizes the possibility of exploiting these parts as a raw material in the production of valuable nutraceuticals and pharmaceuticals, despite their previous characterization as waste. The findings acquired will be valuable when choosing pepper variety for cultivation, well as in the determination of the genotypic and phenotypic variations of pepper from our country and from the different regions in the world. The stalk separated from the fruits of red hot pepper variety *Capsicum annuum* L., ssp. *microcarpum longum conoides*, convar. Horgoshka, cultivated in the Republic of Macedonia can be a promising source of natural valuable products improving the efficiency of resources utilization. The differences obtained concerning the quantity of extractives, fats and content of capsaicinoids, carotenoids and volatile compounds emphasize the need of stalk particle size decrease to 0.25 mm. TGA–DTA/DCS/FTIR analysis show that the grinded red hot pepper stalk with different particle size could be potential renewable, cellulose based, micro-reinforcing filler in biocomposites for industrial and biotechnology application. Stalk with higher particle size (1 mm and higher) are more convenient for composites with improved mechanical behavior because they will be able to take the load. Lower sizes of stalk particles (0.5 and 0.25 mm) would be suitable for barrier/filtration systems since they could be incorporated on macromolecular level in the polymer matrix. The thermogravimetric study of the stalk will be very important to better understand the thermal degradation behavior of natural fibers in relation to polymers obtained by the nanocomposite processing. The findings acquired will be valuable in the future utilization of the stalk as feed and food supplements or polymer matrix ingredient, as well as renewable energy feedstock.

Extraction of oleoresins. In the process of solid-liquid extraction of the placenta from the red hot pepper with ethanol using a technique of maceration in a thermostatic water bath, to obtain the satisfied quantity of extract with high amounts of capsaicin and capsanthin, the extraction at temperature of 40 °C to 50 °C, time of 180 min and 0.04 g /cm^3 solid and liquid phase ratio is is recommended. At extraction of pericarp, placenta, seed, and stalk of red hot pepper wit petroleum ether (40–60 °C), the bigger particle size enabled to obtain less extract yield. The highest quantities were extracted from seed, while the lowest quantities from stalk. The colour differences increased with decreasing the particle size. The highest colour differences (ΔE = 27.12) were estimated at extraction of pericarp. At extraction of pericarp, placenta, seed, and stalk of red hot pepper with petroleum ether (40–60 °C) and Soxhlet method, the bigger particle size enabled to obtain less extract yield. The highest quantities were extracted from seed, while the lowest quantities from stalk. The colour differences increased with decreasing the particle size. The highest colour differences (ΔE = 27.12) were estimated at extraction of pericarp. The influence of the pressure (20, 50 and 100 bar) on the yield of the extracts obtained in the extraction with subcritical propane is insignificant. In the extracts obtained with propane high quantities of colour compounds were extracted, while with supercritical CO_2 the extract are richer with capsaicin and dihydrocapsaicin. At extraction with SCO2 the highest amount of extract were obtained from seed samples. The smaller particle sizes favourised the extraction process by obtaining bigger quantities of extract with satisfactory amounts of bioactive compounds.

Formulation of oleoresins. The extracts obtained from placenta, seed and stalk as an inedible part of red hot pepper can be reutilized as a source for formulations as nanoemuslions, edible films and byopesticides. The prepared formulations were with satisfactory quality parameters.

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