Ultrazvučna ekstrakcija karotenoida iz kore naranče

Marović, Roko

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Roko Marović
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ULTRASOUND ASSISTED EXTRACTION OF CAROTENOIDS FROM ORANGE PEEL

Experimental work for this Graduate thesis was done at the Faculty of Pharmacy, University of Seville, Spain. The thesis was made under guidance of Prof.dr.sc. Antonio J. Melendez-Martinez and with the help of Ana Mª Benitez Gonzalez.

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ULTRAZVUČNA EKSTRAKCIJA KAROTENOIDA IZ KORE NARANČE

Roko Marović, 995/N

Sažetak:U današnje su vrijeme gubici prehrabenih sirovina kao nusprodukata tijekom procesa prerade i obrade veliki problem, a s obzirom na njihovu najčešće visoku nutritivnu vrijednost, zaista je šteta to ne iskoristiti. Svjetska raspostranjenost, dostupnost te popularnost konzumacije naranče i proizvoda od naranče, čine koru od naranče značajnim prehrambenim ostatkom koji obiluje prehrambenim vlaknima te brojnim bioaktivnim komponentama, kao što su polifenoli te karotenoidni spojevi. Upravo su karotenoidni spojevi zahvaljujući svojim svojstvima, između ostalog antioksidacijskim, povezani s brojnim pozitivnim učincima po ljudsko zdravlje. U pogledu njihove ekstrakcijeiz kori naranče, cilj je ovog rada bila optimizirati uvjete ultrazvučne ekstrakcije karotenoidnih spojeva. Utvrđen je pozitivan učinak povećanja amplitude, produljenja vremena ultrazvučnog tretmana te većeg početnog udjela uzorka u otapalu u svrhu većeg prinosa ekstrakcije.

Ključne riječi:*ekstrakcija, karotenoidi, kora naranče,ultrazvuk*

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1.Doc.dr.sc. Sven Karlović

2. Prof.dr.sc. Suzana Rimac Brnčić

3. Prof.dr.sc. Antonio Melendez-Martinez

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Roko Marović, 995/N

Abstract: Nowadays, the loss of raw food materials as a by-product during the processing process is a big problem, and considering their usually high nutritional value, it is really a mistake not to utilize it. The worldwide distribution, availability and popularity of the consumption of oranges and orange products make the orange peel a significant dietary residue that abounds in dietary fiber and numerous bioactive components such as polyphenols and carotenoid compounds. Due to their antioxidant properties, carotenoids have been associated with numerous positive effects on human health. Regarding extraction from orange peel, the purpose of this work was to optimize the conditions of ultrasound assisted extraction of carotenoid compounds. A positive effect of extraction yield with increase in amplitude power, sonication time and sample to solvent ratio is established.

Key words: *ultrasound,extraction, orange peel, carotenoids*

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Mentor: Suzana Rimac Brnčić, Full Professor

Antonio Melendez-Martinez, Associate Professor, University of Seville

Technical support and assistance: Ana M^a Benitez Gonzalez, University of Seville

Reviewers:

1.PhD. Sven Karlović, Assistant Professor

2.PhD. Suzana Rimac Brnčić, Full Professor

3.PhD. Antonio Melendez-Martinez, Associate Professor, University of Seville

4. PhD. Marija Badanjak Sabolović, Assistant Professor (substitute)

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CONTENTS

1. INTRODUCTION

Orange is one of the most popular citrus fruit and highly consumed all over the world. Orange fruit is grown in more than 130 countries and has become relevant good in terms of global agriculture and it takes 10,5 % of the world fruit production (Rezzadori et al., 2012; Milind and Dev, 2012). From the nutritional point of view, orange has high nutritional value, considering that is excellent source of vitamin C, flavonoids, phenolic compounds and pectins (Milind and Dev, 2012). All those nutrients mixed together exhibit synergistic effect which is responsible for many health benefits of human body associated with orange consumption. However, orange may have even stronger positive influence, but unfortunately during consumption, orange peel is mostly discarded, even tough it contains significant amounts of desirable components, including pectin and abundance of phytochemicals, recognized as an powerful antioxidants, among which phenolic components and carotenoid pigments stand out (Rafiq et al. 2018). Instead of negligence, due to its low cost and easy availability, orange peel as a waste has a big potential capable of offering low-cost bioactive compounds. Recent scientific studies has shown functional properties of orange peel that might be useful, especially those due to carotenoids, pigments with a strong antioxidant effect. For years, scientist has been trying to extract carotenoids from orange peel, using several conventional methods with substantial shortcomings. Lately, novel „green and innovative“ techniques have been developed, improving extraction efficiency while being more economic and environment friendly. One of them is ultrasound assisted extraction (UAE), outstanding method, which is becoming more and more popular due to its properties, such as reduction of extraction time, reduction in energy used, reduction of solvent used, reduction of unit operations, environmental impact that all finally contribute to better extraction efficiency.

The aim of this work was to optimize parameters of ultrasound assisted extraction such as sonication time, sample to solvent ratio and amplitude to maximize the extraction of total carotenoids from orange peel. Moreover, the present study was undertaken to compare the effect of using different extraction solvents to extract carotenoids from orange peel.

2. THEORY

2.1. Oranges

Orange is a tasty and juicy citrus fruit, highly consumed all over the world, whether in the natural, peeled form or as a juice. Anatomically, orange is a fleshy and indehiscent simple hesperideous-type syncarpous fruit. Its parts are: epicarp, consisting of the fruit's colorful and outer part; mesocarp, composed by the white portion and located between the epicarp and the endocarp; and endocarp, composed of buds, in which the seeds are inserted (Hamaio Okino Delgado and Francisco Fleuri, 2015). Proper anatomy of citruis fruit is shown in Figure 1.

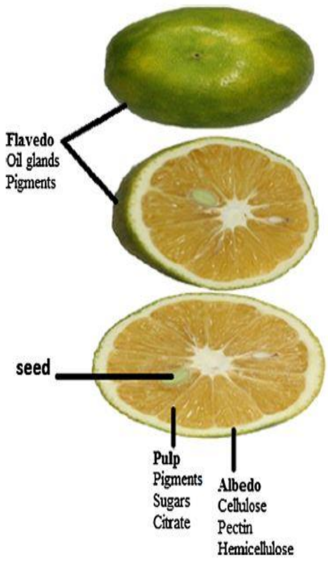

Figure 1. Anatomy of citrus fruit. (Rafiq et al., 2016).

It is botanically known as *Citrus sinensis* and belongs to the family Rutaceae. Orange tree is mostly cultivated and rarely found in the forests (Rezzadori et al., 2012; Milind and Dev, 2012). The origin of orange is controversial, but generally it is considered that it firstly occured int he Malay Archipelago, China. In 11th century, orange was introduced in Europe, precisely in Italy. However, in the begginings, orange was considered as a bitter fruit and primarily grown for medical purposes, but by the time it spread throughout Europe and had become very edible and liked fruit. It was brought to the South America with the discovery of continent and very soon it had become widely cultivated, because at that time, it was considered as an antidote to scurvy (Hamaio Okino Delgado and Francisco Fleuri, 2015; Milind and Dev, 2012). Nowadays, oranges are one of the most important goods in terms of global agriculture, with total production reported to be around 120 million tons, which is 10.5 % of the world fruit production. Orange trees are widely cultivated in tropical and subtropical climates, due to its good taste and nutritional and medicinal value. Orange fruit is grown in more than 130 countries, but main productions regions are found in Spain, Italy, India, China, Brazil, USA… For example in Brazil, orange production represents 49 % of the total fruit production in the country. Moreover, Brazil is responsible for around 53 % of total global orange juice production and for 80 % of the international trade (Rezzadori et al., 2012; Milind and Dev, 2012). It is a major crop with many relevant bioactive compounds (Rodriguez-Concepcion et al., 2018). Orange is also widely used in production of orange juices which represents one of the most globally accepted fruit products and their consumption is increasing worldwide. Carotenoid pigments are responsible for the characteristic colour of both the peel and the pulp of most varieties of ripe oranges, although the colour of so-called blood oranges is different due to anthocyanins also for the reddish colour of the juices obtained from them (Melendez-Martinez et al., 2007). From the nutritional view, oranges are rich source of vitamin C, flavonoids, phenolic compounds and pectins, while also containing solid amounts of many micronutrients, which average quantities are shown in Table 1. The main flavonoids that are found in orange are hesperidine, narirutin, naringin and eriocitrin. One average orange might provide 116 % of the daily requirement for vitamin C, well known water – soluble antioxidant, which fights against free radicals in the body and in that way prevents damage to the cells and tissues. Vitamin C is also necessary for the proper functioning of immune system and might reduce risk of getting cold, cough and reccurent ear infections. Limonene, one of the orange main bioactive chemicals reduces the risk of mouth, skin, lung, breast, stomach and colon cancer. Another constituent, hesperidin also has anti-carcinogenic acitivites in various *in vivo* studies and beta cryptoxanthin may significantly lower risk of developing lung cancer. Besides anti-carcingenic effect, oranges, consumed in a form of orange juice, might reduce the risk of kidney stones formation and prevent from develpoment of the ulcers (Milind and Dev, 2012).

Table 1. Nutritional value of Orange (Milind and Dev 2012).

Elements	Amount (100g)
Energy	192 kJ
Carbohydrate	11.54 g
Sugar	9.14 g
Fat	210 mg
Protein	700 mg
Dietary fiber	2.4 g
Thiamine	100 µg
Riboflavin	40 µg
Niacin	400 µg
Pantothenic acid	250µg
Vitamin B6	5 µg
Folate	17 µg
Vitamin C	45µg
Calcium	43 µg
Iron	90 µg
Magnesium	10 µg
Phosphorus	12 µg
Potassium	16.9µg
Zinc	80 µg

2.2. Orange waste and byproducts

Citrus fruits are highly consumed worldwide and during consumption orange peel is the most often discarded as a waste, which is rich in secondary components, considered to have substantial antioxidant activity in comparison to the edible parts of the fruit. Global production of citrus fruit has siginificantly increased recently and therefore, a large amount of fruit peel is produced every year. The primary waste, citrus peel is usually dried, mixed with dried pulps and sold as cattle feed (Rafiq et al., 2018). Citrus peel has two parts, the core is composed of mesocarp, which is also called albedo and it constitutes the largest byproduct volume produced in orange juice processing. It corresponds to 42 % of the total of the fruit. It contains flavones, pectin, cellulose fiber and minerals such as iron and chromium. The epicarp contains a high concentration of carotenoids and limonene, which are among the substances with higher concentrations in citrus essential oils (Hamaio Okino Delgado and Francisco Fleuri, 2015). Orange waste contains 16.9 % soluble sugars, 9.21 % cellulose, 10.5 % hemicelluloses and 42.5 % pectin. The orange peel represents around 20 – 30 % of the total orange mass, and accordingly abundant, cheap and available biomass. The orange peel consists of cellulose, pectin, hemicellulose, lignin, volatile essential oils, carotenoid, phenolic components. The soluble sugars presented in orange peel are glucose, fructose and sucrose and insoluble polysaccharides incorporated in cellular wall are made of pectin, cellulose and hemicellulose. Pectin and hemicelluloses contain significant amounts of galacturonic acid, arabinose and galactose and also contain small amounts of xylose, rhamnose and glucose. (Baaka et al. 2017; Rezzadori et al., 2012). The orange fruits peel represents a rich source of carotenoidic pigments, especially β-carotene, β-cryptoxanthin, violaxanthin and lutein, that are important A vitamin precursors and provides numerous benefits for human health. Their chemical structures are shown in Figures 2, 3, 4 and 5. Orange peel carotenoids concentration and composition varied due to different plant variety and growth condiditions which orange is exposed to. It was acknowledged that the β-carotene extracted from orange peel showed a higher bioavailability that the synthetic one, substantial economically advantages as well (Dumbrava et al., 2010). Recent scientific studies has shown functional properties of citrus byproduct that might be useful. Due to its low cost and easy availability, fruit residues have potential to be nutraceutic resources, capable of offering low-cost, nutritional dietary supplements. Instead of negligence, waste can be recycled as added value to food supplements, providing advantageous dietary fiber and polyphenols, which are known for ability to prevent human body from a wide range of diseases caused due to oxidative stress. The extracts from fruit peel hold promise in food industry as sources ofbioactive compounds. In addition, an established use of the citrus peel would also help alleviate pollution problems causedbecause of the poor disposal of such residues (Rafiq et al. 2018). Hydroxylated polymethoxyflavones and methylated flavonoids are present in sweet orange peel showed cytoprotective effects against oxidative stress which improves the maintenance of cell normal redox status. Citrus peel is source of phenolic compounds, including phenolic acids, polymethooxyflavones and glycosyflavanones and many other phytochemicals which are presented in Table 2. Polyphenols as an antioxidants exhibit functional and nutraceutical activity. Lypolytic enzymes, obtained from sweet orange peel, including esterases and lipases, differs in affinity to substrates: lipases catalyze the hydrolysis of triglycerides composed by long-chain fatty acids; esterases preferentially hydrolyze „simple“ esters and triglycerides fromed by short-chain fatty acids (Hamaio Okino Delgado and Francisco Fleuri, 2015).

Table 2. Phytoconstituents of orange (Milind and Dev, 2012).

Sr.	PHYTOCONSTITUENTS	PLANT PART
1.	Flavone glycosides; Neohesperidin, Naringin, Hesperidin, Narirutin, Triterpene; Limonene, citrol Pigment; Anthocyanin, β-cryptoxanthin, Cryptoxanthin, Zeaxanthin and Rutin, Eriocitrin, Homocysteine Polymethoxylated flavones; Tangretin and Nobiletin Flavonoids; Citacridone, Citbrasine and Noradrenaline	Fruit Peel
2.	Terpenoid; Linalool, β-elemene	Leaves
3.	Triterpenes; Limonene	Flowers
4.	Vitamins: B1, B2, B3, B5, B6 and Vitamin C Minerals: Calcium, Iron, Magnesium, Zinc, Phosphorus, Potassium	Fruits

During orange juice production, around half of the orange weight is not turned into juice, but left as a residual waste in form of peel, pulp, seeds, orange leaves and even whole orange fruits which do not reach quality requirements. Moisture content in waste is around 82 %. However huge amounts of waste are handled by using in animal feed or are burned, which is environment unfriendly method, because it can negatively affect the soil, the ground and superficial waters. Orange waste spilled into the environment is called „yellow water“ and has high pollution potential. Another solution might be implementation of new recovery waste methods, for example, through the production of organic fertilizers, pectin, bio-oil, essential oils, antioxidant compounds, or as a substrate for a production of microbial proteins, organic acids, ethanol, enzymes, biologically active secondary metabolites and adsorbent materials. Some parts of the citrus waste has already been extracted for similar purposes, for instance essential oils obtained from orange peel, is used as an ingredient in foods, beverages, cosmetics and perfumes, aromatic essences; d-limonene is use in production of ink and solvents; the bran of citric pulp is use in production of animal feed; orange pulp is used in production of foods and beverages (Rezzadori et al., 2012).Furthermore, natural dyes can be extracted from the food industry waste and can be a significant substitute and alternative to synthetic. Carotenoids dye isolated from orange peels has good properties and can be use din cotton fabrics. The essential oil is the most valuable byproduct in orange juice processing, and it is widely used due to its essence, coloration and antimicrobial properties. Its concentration is about 0,3 % of the total fruit weight and it is extracted by mechanical pressure. The oil storage glands are concentrated in the outer part of the epicarp (frit). So, in order to facilitate the oil extraction process, during the juice extracting process, the epicarp fraction is separated from the rest of the peel. The orange essential oil consists of a mix substances including terpenes, which are compounds that are found in high concentration rates. They also refer to hydrocarbons formed by the condensation of isoprene units. They share their origin with fatty acids and are classified as unsaponifiable lipids (Hamaio Okino Delgado and Francisco Fleuri, 2015).

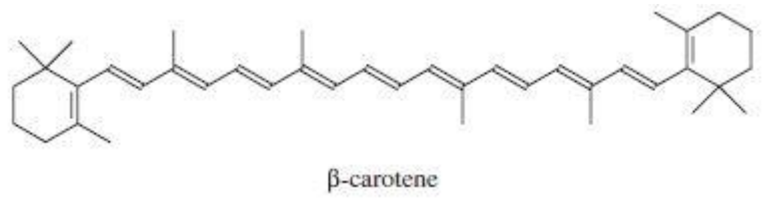

Figure 2. Beta-carotene structure(Melendez-Martinez et al., 2007).

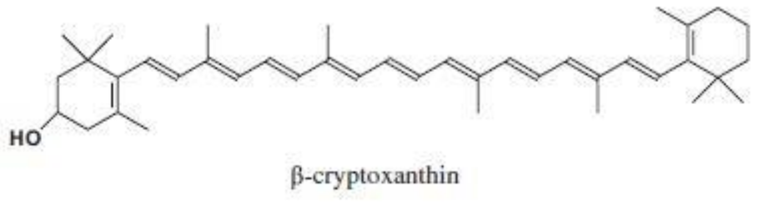

Figure 3. Beta-cryptoxanthin structure (Melendez-Martinez et al., 2007).

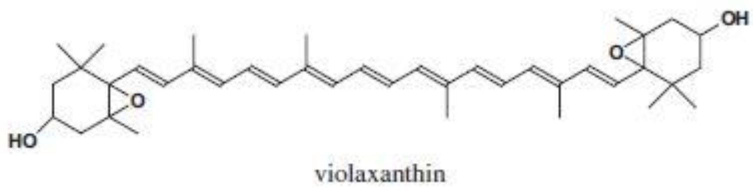

Figure 4. Violaxanthin structure(Melendez-Martinez et al., 2007).

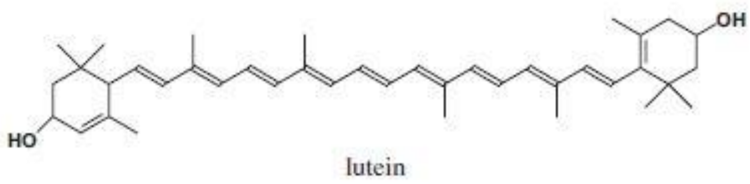

Figure 5. Lutein structure (Melendez-Martinez et al., 2007).

2.3. Carotenoids

Carotenoids are the most widespread class of isoprenoid pigments and have interested many researchers, because of their commercially desirable properties, such as their natural origin, wide distribution, structural diversity, very important biological function, null toxicity and high versatility. Carotenoids are present in chromoplasts and chloroplasts and can be biosynthesized in the plant, by algae, yeast, fungi and photosynthetic bacteria, mostly in life forms where photoysinthesis occurs, but in certain cases, carotenoids are present in non-photosynthetic yeasts, moulds and bacteria. All these organisms sytnhesize carotenoids from fundamental organic metabolic building blocks. (Rubashvili et al., 2018; Singh et al., 2015). Animals (except some species of aphids) can not synthesize carotenoids, hence they depend on dietary source. In animals, carotenoids have relevant roles, including: i) ornamentations; ii) protection against lung, head, neck and prostate cancer, most likely due to their potent antioxidant properties mediated by oxidizing the superoxide radical anion; iii) in the modulation of the immune system, growth factors and intracellular signaling pathways; iv) the regulation of cell differentiation, cell cycle and apoptosis; v) photoprotection against UV radiation; and vi) as precursors for the visual pigment retinol know as vitamin A (Saini and Keum, 2018). More detailed roles are shown in Figure 6.

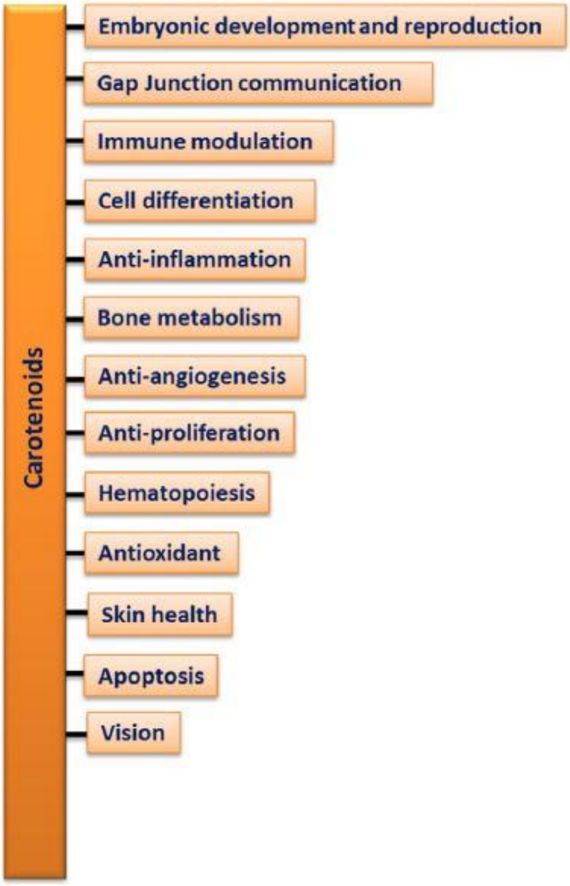

Figure 6. Biological activities and properties of carotenoids in animals (Saini et al., 2015)

Carotenoids are lipid-soluble tetraterpenoids with 40 carbon atoms in their structure, having conjugated double bonds. It means they are formed from eight C5 isoprenoid units joined head-to-tail, exceptat the center where a tail-to-tail linkage reverses the order, resulting in a symmetrical molecule (Zahgdoudi et al., 2015). However even though C40 are mostly found carotenoids in nature, some of them are shorter or longer. For example decaprenoxanthin, a C50 carotenoid has ten C5 isoprenoid units (Rodriguez-Conepcion et al., 2018). In Figure 7. It can be noticed, most of naturally occurring carotenoids are all-*trans* configuration but they get isomerised to *cis* configuraiton due to conjugation during processing or when they are exposed to certain environmental conditions, for instance, intensive exposure to light and/or heat . Even though trans- from is the most stable, carotenoids are prone to geometrical isomerization, so they can also naturally occur in *cis* configuration, such as 15-*cis*-phytoene.Furthermore, for some of biologically important carotenoids, cis-form is proper for their functionality in light-harvesting complex or in the synthesis of hormones derived from carotenoids(Melendez-Martinez et al. 2013; Rodriguez-Conepcion et al., 2018). It is not all the same whether carotenoids are present in *cis* or *trans* isomer. Moreover, isomer form strongly affects their behaviour, changing their functions in biological tissues, including bioavailability, vitamin A activity, stability towards electrphiles and specificity for cleavage enzymes (Melendez-Martinez et al., 2013). Isomerisation grade is determined by time and strength of the heat. Carotenoids' trademark is extended conjugated double-bond system, which constitutes thelight absorbing chromophore that provides the visible absorption spectrum. Every type of carotenoid has unique absorption spectrum, hence it is useful in manner of their identification and quantification. Moreover, it provides attractive colours to various fruits and vegetables as well. For instance, lycopene is source of red colour to several fruits and vegetables, contain 11 conjugated and 2 unconjugated double bonds (Singh et al., 2015; Zahgdoudi et al., 2015). To date, more than 700 natural carotenoids have been identified and the number is still increasing every year. According to structure, carotenoids are classified into two groups: oxygenated, called xanthophylls (e.g., β-cryptoxanthin, lutein, zeaxanthin, canthaxanthin) and non-oxygenated, called carotenes (e.g., α-carotene, α-carotene, lycopene) (Rubashvili et al., 2018; Zahgdoudi et al., 2015). Common occuring xanthophylls and carotenes are shown in Table 3.

Table 3. Common carotenes and xanthophylls in foods and natural sources (Rodriguez-Concepcion et al., 2018).

Type	Carotenoid	Natural occurence
Carotenes	α -carotene, γ-carotene, δ-carotene, ε-carotene, ζ- carotene β-carotene	Fruits and vegetables, especially in carrots, sweet potato, palm tree fruit. Rose hips are good source for γ-carotene.
	lycopene, neurosporene	Tomato, water melon and rose hips.
	phytofluene, phytoene	Carotenoid-rich fruits, flowers and carrot.
Xanthophylls	antheraxanthin	Fruits and green vegetables. Anthers and petals of many yellow flowers.
	astaxanthin	Fish (salmon) and crustaceans (e.g. lobster).
	Bixin, norbixin	Annatto (*Bixa orellana*) seeds.
	canthaxanthin	Fungi, cyanobacteria and green algae.
	Capsanthin, capsorubin	Pepper ripe fruits.
	crocetin	Saffron stigmas.
	Cucurbitaxanthin A	Pumpkin flesh.
	lactucaxanthin	Lettuce leaves.
	lutein	Green fruits, vegetables, flowers and cereal grains (wheat). Also in egg yolk.
	Violaxanthin, neoxanthin	Green fruits, vegetables and flowers.
	Luteoxanthin, auroxanthin, mutatoxanthin	Vegetables and fruits processed under acid conditions and fermentation.
	rubixanthin	Rose hips.
	zeaxanthin	Corn, potatoes, red pepper. Alson in egg yolk.
	β-cryptoxanthin α-cryptoxanthin	Seeds (corn), flowers and fruits: sweet orange, rangerine, mango, papaya, persimmon, pepper.

Xanthophylls contains oxygen as a functional group and are synthesized within the plastids and chromatographically separated from other carotenes due to different polarity. Among all other xanthophylls, β-cryptoxanthin is the only one that has pro-vitamin A activity for mammals. Xanthophylls do not require light for synthesis, hence they are present in all young and etiolated leaves and have a relevant function as accessory pigments by capturing certain wavelength of sunlight which are not absorbed by chlorophyll. Carotenes opposite to xanthophylls, they do not have functional group. Carotenes help photosynthesis by transmitting the light energy, they absorb from chlorophyll and protect plant tissues by absorbing the energy from singlet oxygen as well (Singh et al., 2015). Carotenoids in plants are present in free form or esterified with fatty acids. Unesterified carotenoids are present in green leaves, while ripened fruits contain esterified carotenoids along with fatty acids. Esterification does not change the chromophore properties of the carotenoid, but influences the chemical and biological propertiesby changing its immediate environment. Properties also depend on fattyacid that is bound to the carotenoidmolecule. Esterification improves carotenoid storage, as a matter of fact it helps these highly lipophilic molecules to incorporate into lipid rich plasto-globules. It is assumed that esterification is the natural, evolutionaly made biological mechanism toprotect triacylglycerols, unsaturated lipids, and other light sensitive compounds from photooxidation (Saini et al., 2015).

Quality of fruits and vegetables are dependent on present carotenoids. Colours of fruits, vegetables, flowers, leaves and also in case of certain insects, fish, birds and crustaceans are due to carotenoids, for instance colours in tomatos, peppers which are red and pink colour of salmon and flamingos (Singh et al. 2015).Carotenoid bioavailability is influenced by a combination of dietary and physiological factors and varies depending on the type of carotenoid, surrounding food matrix and host-related factors. The observed variability in absorption, distribution, metabolism and extraction can be attributed to different host-related factors, including age, weight, disease state, physical activity, alcohol use, smoking habits, drug intake, or genetic aspects, but also to microbiota, presence of parasites and concentration of enzymes and other components involved in digestion (Rodriguez-Concepcion et al., 2018).

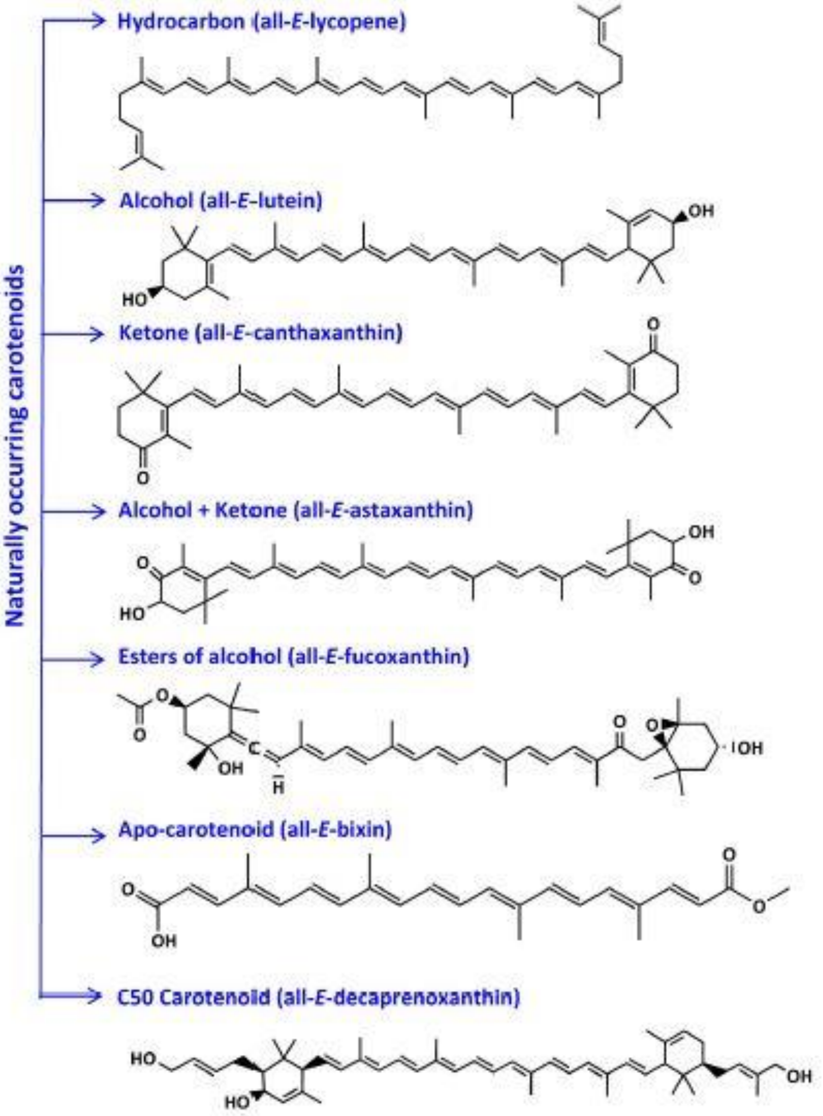

Figure 7. Naturally occuring carotenoids (Saini and Keum, 2018).

The type and availability which is compared in Figure 8., of carotenoids in fruits and vegetable can be assumed by their color, such as yellow-orange vegetables and fruits are generally rich in β-carotene and the α-carotene. α-Cryptoxanthinand zeinoxanthin can be found in orange fruits, such as mandarin,orange, and papaya. Similarly, lycopene pigment is crucial component of tomatos and tomatoproducts. Lutein (nearly 45%) and β-carotene (25–30%) followed byviolaxanthin (10–15%), and neoxanthin (10–15%) are majorforms of carotenoids in green leafy vegetables. α-Carotene, β-cryptoxanthin, zeaxanthin,antheraxanthin, and lutein 5,6-epoxide (luteoxanthin) are alsofoundin green leafy vegetables, but in a low quantity. β-carotene is generally dominating compared to its geometric isomer α-carotene in most of the plants. Substantial high contents of α-carotene can be found in a limited number of fruits and vegetables, including carrots, pumpkin,sweet potato, palm oil fruit and dark green vegetables,like green beans, spinach and broccoli. Knowledge on carotenoid composition in different edibleparts and cultivars might be be useful to nutritional experts for the selectionof nutrient-rich plants for food fortification and proper diet recommendation (Saini et al., 2015). Humans can intake about 50 carotenoids with their diet, mainly through fruits and vegetables. But after absorption the number drops to around 20 from which, b-carotene, α-carotene, lutein, zeaxanthin, b-cryptoxanthin andlycopene are the mostly found in blood plasma (Rodriguez-Concepcion et al., 2018). For example, the presence of epoxides of xanthophyllshave not been reported in plasma (Melendez-Martinez et al., 2013). In fact a-Carotene, β -carotene, lutein and lycopene constitute 90% of total carotenoids of the human diet, even tough there have been found 34 different carotenoids in human serum and milk (Singh et al., 2015).

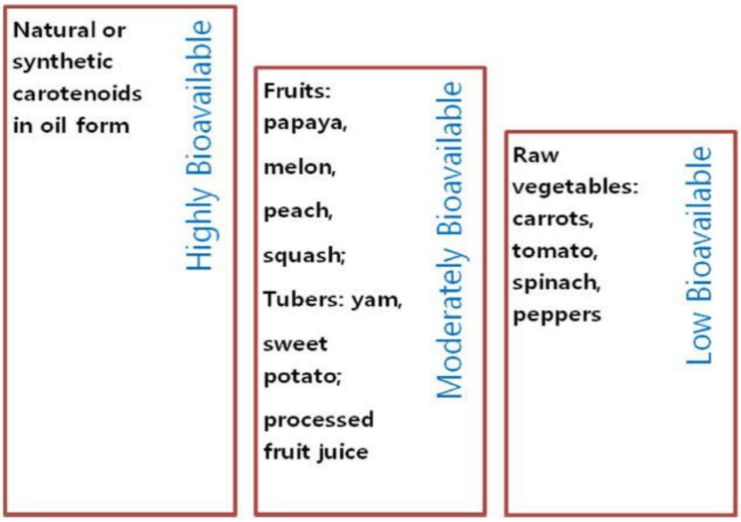

Figure 8. Comparative bioavailability chart of carotenoids from fruits and vegetables (Saini et al., 2015)

Carotenoids have been associated with beneficial impact on human health. They show antioxidant activities, which may prevent degenerative diseases, such as cardiovascular, dermatological, renal, pulmonary diseases, the oxidative damages that are specific to ageing phenomena, cataract and macular degeneration, toxic liver damage, metabolic syndrome, sepsis, autoimmune disorders, diabetes and several types of cancer, especially, prostate and digestive-tract tumors. Also, it was determined that the antimutagenity is mostly associated with hydrocarbon carotenoids fractions (α-carotene, β-carotene, lycopene) and with xanthophylls (lutein, β-cryptoxanthin), therefore, their supplementation can increase CD4 counts in HIV-infected patients. Retinol and several carotenoids (β-carotene, β-cryptoxanthin, zeaxanthin, lutein, capsorubin, capsanthin, lycopene and capsanthol) contribute to cytoprotective effect on injury of gastric mucosa (Rubashvili et al., 2018). Lately, studies has been paying attention on carotenoids and retinoids and their role in the control of lipid and energy metabolism. Retinoids exhibit anti-adiposity effect due to modulation of many processes, including adipocyte differentiation, lipogenesis, thermogenesis, lipolysis and fatty acid oxidation in tissues such as liver, white and brown adipose tiussues and skeletal muscle. Hence, carotenoids implications for disorders such as obesity, diabetes, nonalcoholic fatty liver disease and atherosclerosis should definitely be considered (Rodriguez-Concepcion et al., 2018). Epidemiological studies showed that the consumption of diets rich incarotenoids is associatedwith a lower incidence of cancer, cardiovasculardiseases, age related macular degeneration and cataract formation. Deficiency of carotenoids results in clinical signs of conjunctiva and corneal aberrations including,xerophthalmia, night blindness, keratomalacia, corneal ulceration, scarring,and resultant irreversible blindness. Futhermore, deficiency of provitamin A carotenoids leads to visiondisabilityin human and increased mortality due to a weakened immune system (Saini et al., 2018).

2.4. Ultrasound assisted extraction (UAE)

Extraction has been known since the discovery of fire. Egyptians and Phoenicians, Jews and Arabs, Indians and Chinese, Greeks and Romans, and even Mayas and Aztecs, all used extraction and distillation processes for perfumes, cosmetics or food. Nowadays, extraction is widely used everywhere, in a production line in food, pharmaceutical, cosmetic, nutraceutic, or even bioenergy industries (Chemat et al., 2017).

In last few decades a lot of effort has been put in terms of developing better extraction methods for carotenoids, but the efficiency rate of recovery still remains relatively low. Firstly, it is due to various physical and chemical obstacles from complex food matrix that prevent the mass transfer of carotenoids during extraction. Secondly, simultaneous extraction of different carotenoids might be complicated because carotenoids differs in polarity. Thirdly, carotenoids are very sensitive and can easily oxidize so its exposure to the heat, light, acids and long extraction times should be controlled and limited (Saini and Keum, 2018).

Extraction is the first step in the recovery process of bioactive compounds from a solid plant material using solvents and there are several methods than can be used in order to obtain compounds from peel wastes. They includeconventional solvent extraction, alkaline extraction, resin-based extraction,enzyme-assisted extraction and subcritical water extraction. However, many of those have concrete disadvantages, such as degradation of compounds which can occur in conditions of high temperaure, light and air exposure and long extraction time. It is already known that conventional methods are time-consuming and have low efficiency as well (Zahgoudi et al., 2015; Arshadi et al., 2016; Prakash Marane et al., 2017). These shortcomings have led to the development of new sustainable ‘‘green and innovative’’ techniques in processing, pasteurization and extraction, which typically require less time, water and energy(Chemat et al., 2011). Moreover, they do not contain hazarodus organic solvents or at least require reduced quantity, consequently extracted compounds are free from unwanted dangerous chemicals (Singh et al., 2015). Green extraction ofnaturals products could be a new concept to meet the challengesof the 21st century, in terms of to protect both, the environment and consumers protection while enhancing competition of industriesto be more ecologic, economic and innovative(Chemat et al., 2017).As a result, a novel techniques have been realized recently, including ultrasound assisted extraction (UAE),microwave assisted extraction, supercritical fluid extraction and pressurized fluid extraction (Marić et al, 2018). Among all of them, UAE is the simplest and economically the cheapest method of extraction (Dey and Rathod, 2013). Compared with conventional solvent extraction methods, ultrasound extraction can improve extraction efficiency and extraction rate, reduce extraction temperature and increase the selection ranges of the solvents(Sun et al., 2011).

UAE is outstanding technique, considering its simplicity and extraction rate, hence it consumes less energy, time and materials, while producing more pure products at higher yields (Ofori-Boateng and Lee, 2013). As a result, application of UAE is becoming more and more popular and it has been widely used for the increasment in rate and yield of extraction of nutritional compounds, such as proteins, lipids, fiber, flavoring, essential oils and phytochemicals, which are all constituents in plant materials (Purohit and Gogate, 2015; Sun et al., 2010). Ultrasound uses physical and chemical phenomena that are significantly different compared with those applied in conventional extraction, processing or preservation techniques. Because of that, in Figure 9. it is shown, in comparison to classical method, UAE has substantial advanatages in terms of productivity, yield and selectivity, efficent processing time, enhanced quality, reduced chemical and physicalhazards, and is environmentally friendly (Chemat et al., 2011).

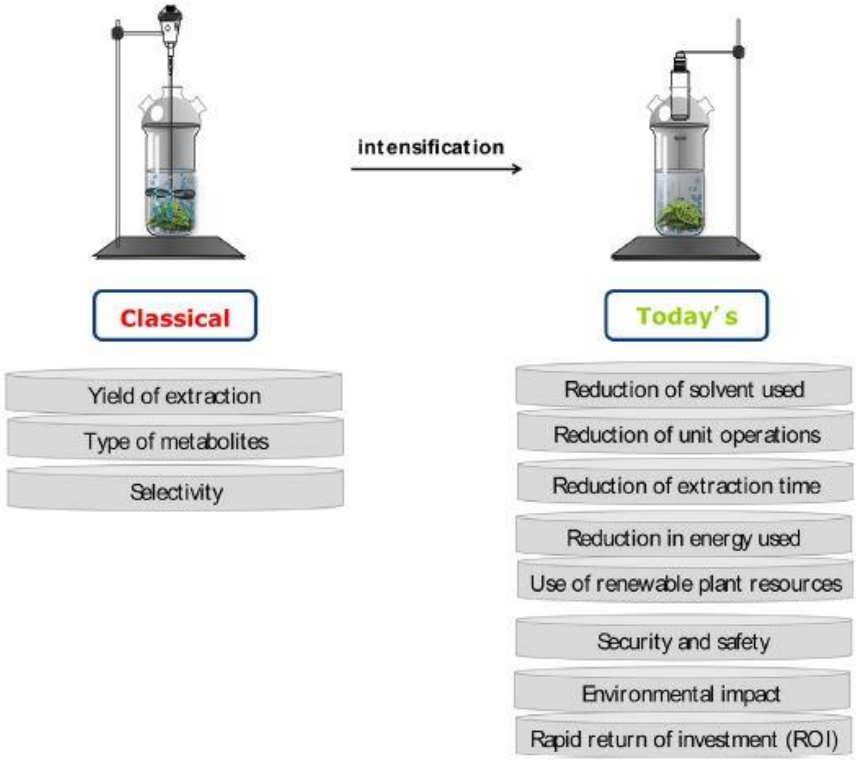

Figure 9. Comparison between classical and today's extraction (Chemat et al., 2017).

Ultrasound is a non-thermal technology which is more suitable for enhancing the extraction of thermally unstable compounds by reducing risk of degradation (Prakash Maran et al., 2017).Ultrasounds are successively implemented in plantextraction field and is well known to have a greateffect on the rate of various processes in the chemical andfood industry. Ultrasound reduces time of full extractions while having high reproducibility, reducing the consumption of solvent, simplifying work, giving higher purity of the final product, eliminating post-treatment of waste water and consuming only a little bit of the fossil energy normally needed in significantly higher quantity for a conventional extraction method such as Soxhlet extraction, maceration or steam distillation. Ultrasound waves alter physical and chemical properties of the plant material they are interacted with. UAE is a clean method that does not require large amounts of solvent and its working time is less in comparison to other methods (Chemat et al., 2011). Its improvements in extractions are due to acoustic cavitation that consists on the formation, growth and collapse of microbubbles inside a liquid submitted to high frequency sound waves. This collapse happens simultaneously with localized extreme pressures and temperatures, strong acoustic streaming, high shear stress near the bubble wall, microjets near the solid surfaces due to asymmetric collapse of bubbles and turbulence. As it is shown in Figure 10. these mechanical effects of ultrasound clearly obstruct cellular tissue while providing a better penetration of solvent into the cells which results in releasment of compounds from their matrices (Luengo et al., 2014).

Cavitation bubbles are able to cause the rupture of the plant cell and reduce the partcile size while easing the flow of solvent into the extractable sample which finally enhance mass transfer rate (Ofori-Boateng and Lee, 2013; Purohit and Gogate, 2015).

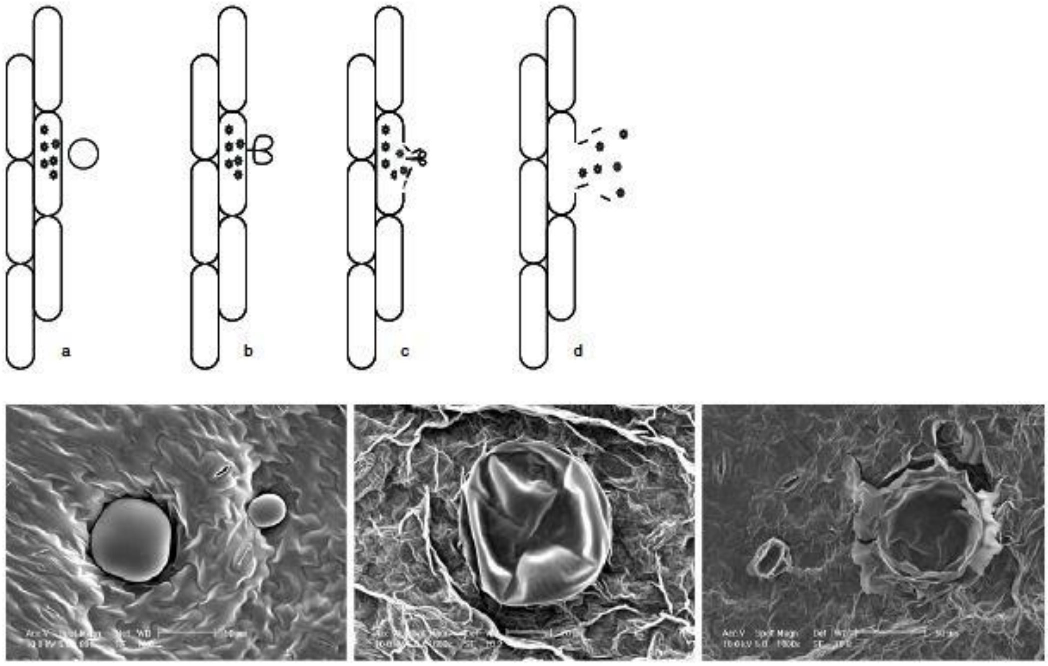

Figure 10. Cavitation bubble collapse and plant material releasing (Chemat et al., 2011).

All these effects are achieved by combination of several mechanisms, including fragmentation, erosion, sonocapilary effect, sonoporation, local shear stress and maybe detexturation. Combining mixing effects to physical impacts of ultrasound on raw material may explain the enhanced extraction performances of ultrasound (Chemat et al., 2017).

3.EXPERIMENTAL PART

3.1. Materials

About 10 kg of oranges were purchased from local store (Seville, Spain).

Chemicals

- Methanol (reagent grade, ACS, ISO, Scharlau)
- Acetone for analysis (ACS, ISO, ITW Reagents)
- Hexane for HLPLC (VWR Chemicals)
- Ethanol absolute for HPLC (ITW Companies)
- Ethyl acetate (HPLC grade, Fischer Scientific)

Apparatus:

- Knife
- Plastic bags (for sample storaging)
- Analytical mill(A 11 basic, IKA, Germany)
- Weight scale (Sartorius TE 124S, Germany)
- Falcon tubes
- Vortex(Vortex-Genie 2, G-560E, Scientific Industries, USA)
- Sonicator(Q500, Qsonica,USA)
- Centrifuge (5810 R, Eppendorf, Germany)
- Spectrophotometer (Cary UV-Vis, Agilent Techonologies, USA)
- Concentrator (plus 5305, Eppendorf, Germany)
- Manual pipettes
- Spatula
- Microtubes (Eppendorf, Germany)
- Quartz cuvette
- Glass beaker
- Freezer
- Nitrogen gas generator

3.2. METHODS

3.2 1. Sample preparation

Oranges were selected based on their uniformity in shape and color. Orange peels were then carefully cut and separated by a knife, before getting dried via lyophiliziation. Lyophilized orange peels were put into plastic bags which are shown in figure 11. and stored into freezer to prevent oxidation and degradation of active compounds.

Figure 11. Lyophilized orange peels

Pieces of dried peels were then pulverized using analytical mill (A 11 basic, IKA – Analytical mill) to powder form and mixed into uniform lot. Furthermore, approximately 1 gram of powder had been weighted on weight scale (Sartorius TE 124S) and had been put in each of 15 different falcon tubes which looks like one in Figure 12.

Figure 12. Weighing of the orange peel samples for extraction

3.2.2. Ultrasound assisted extraction

Ultrasound assisted extraction was performed with an ultrasonic processor, sonicator Q500 (Qsonica, USA), operating at 20 kHz with maximum power rating of 500 W. It is equipped with generator, converter, sound enclosure, microtip probes and display, where power of amplitude and time can be adjusted and showed while process of sonication. Everything is shown in Figure 13. Triplicates of samples were made for every solvent and each of them were placed in an ultrasonic bath filled with an ice to prevent temperature increase. Sonication was performed with clearly determined parameters: sonication time (1 min); power of amplitude 20 %; probe diameter 13 mm.

Figure 13. Sonication process

Evaluation of the best solvent for the extraction of carotenoids from ground orange peel were conducted in five different solvents: methanol, acetone, hexane, ethanol and ethyl acetate which are shown in figure 14.. For every solvent three repetitions were made. Briefly, about 1 gram of pulverized orange peel was mixed with 25 ml of solvent, placed into falcon tube and shaken by vortex device (Vortex-Genie 2, G-560E) in order to enhance the rate of homogenization in sample.

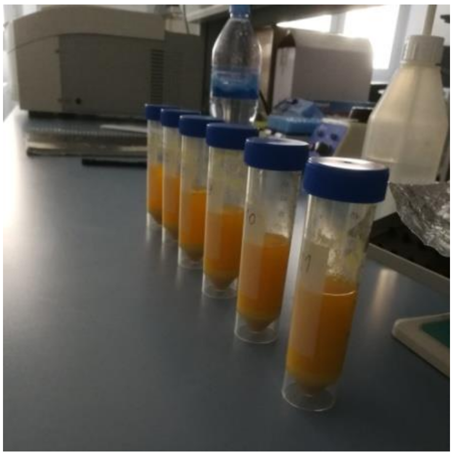

Figure 14. Samples prepared for selection of best solvent for extraction of carotenoids from orange peel

3.2.2. Total carotenoid content

After all samples had been sonicated they were left aside for a few minutes to be sure their temperature dropped to the room temperature levels. Next action was the process of centrifugation in order to do better separation between particles so more total carotenoids could have been extracted from orange peel material.Samples were centrifugedin Centrifuge at velocity of 4000 rpm for 3 minutes at temperature 4 °C is shown in figure 15.

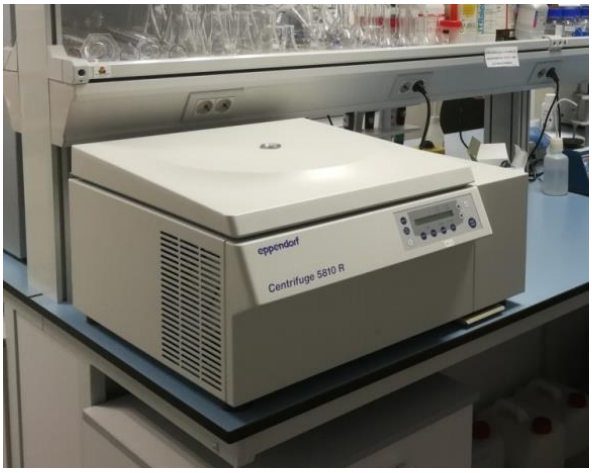

Figure 15. Centrifuge

After centrifugation samples in falcon tubes had two phases, solid precipitate at the bottom and overlying clear liquid, supernatant. Next step was making dilution 1:4, Firstly, 0.5

mL aliquots were carefully taken from supernatant in falcon tubes via manual pipettes and put into plastic microtubes. Secondly 1.5 mL of solvent was added into plastic microtubes. Before spectrophotometric measurement started, device had been turned on for at least 10 minutes, and quartz cuvette was cleansed. Firstly, blind probe was made before measuring of every different triplicate in order to check cuvette clearance and to be sure there are no other interferences. After blind probe was done, aliquote was taken from microtube and was put into quartz cuvette by using micropipette and then placed in device and got measured. Apsorbance was measured at 450 nm. Measurements were made in triplicate.

Concentrations of the total carotenoids were determined spectrophotometrically using the specific extraction coefficients. (Britton, 1995).

$$\text{total carotenoids}\left(\frac{\text{mg}}{\text{g}}(\text{of DW})\right)=\frac{AxY}{2500x100}x\ DF \qquad /1/$$

A= Apsorbance at 450 nm

Y= Volume of the sample (mL)

DF = Dillution factor

DW = Dry weight of sample

From the same sample’s supernatants from which aliquots were taken to microtubes in order to get them prepared for spectrophotometry, another aliquots were taken into another microtubes in order to storage them. Firstly 1 mL of aliquot was taken into microtube and than were drying in concentrator at the temperature 30 °C for 20 minutes which is shown in figure 16.

Figure 16. Concentrator

Finally, when the samples in microtubes were dried they were taken and microtubes get closed under atmosphere of nitrogen gas and kept in the freezer.

3.2.3. Experimental design

After the best solvent had been examined, main part of experimental work was designed in order to figure out what are the best conditions for ultrasound assisted extraction. It includes the same processes as those which were used in preliminary phase, the only methodical differences were periodically changes in weight of ground orange peel, amplitude of sonicator and sonication duration. Solvent volume remained the same, 25 mL. In one batch 36 different assays were done and they are showed in Table 4. The batch was repeated three times.

Table 4. Design of experimental work

Observation	Weight of orange peel (g)	Amplitude of sonicator (%)	Sonication time (min)
1	2.5	20	0.5
2	2.5	20	1
3	2.5	20	2
4	2.5	20	4
5	2.5	30	0.5
6	2.5	30	1
7	2.5	30	2
8	2.5	30	4
9	2.5	40	0.5
10	2.5	40	1
11	2.5	40	2
12	2.5	40	4
13	5	20	0.5
14	5	20	1
15	5	20	2

16	5	20	4
17	5	30	0.5
18	5	30	1
19	5	30	2
20	5	30	4
21	5	40	0.5
22	5	40	1
23	5	40	2
24	5	40	4
25	7.5	20	0.5
26	7.5	20	1
27	7.5	20	2
28	7.5	20	4
29	7.5	30	0.5
30	7.5	30	1
31	7.5	30	2
32	7.5	30	4
33	7.5	40	0.5
34	7.5	40	1
35	7.5	40	2
36	7.5	40	4

3.2.4. Dillution ratio adjustment

Due to considerable differences in initial ground orange peels weight, during preparation of sample for spectrophotometric analysis, certain modificiations in dilution ratio in microtube had been made. For 2.5 gram sample weight, dillution ratio was 1:16 (125 μL of aliquote); for 5 gram sample weight, dilution ratio was 1:32 (62.5 μL of aliquote); for 7.5 gram sample weight, dilution ratio was 1:50 (40 μL of aliquote).

3.2.5. Statistical analysis

The software Statistica 12 (Statsoft UK) was used. In order to examine hypotesis „Amplitude and sonication time effect on extraction yield“, one way and two ways ANOVA have been implemented with level of marginal significance $p < 0.05$ as an conditional assumption. Tukey HSD procedure has been taken for multiple comparison in order to determine differences in means among experimental values. Thus simultaneously has been highlighted what level of amplitude and sonication time had the strongest effect on extraction yield. 3D contour graphs have been made in order to obtain optimal parameters, considering two scenarios, first extraction yield dependence on amplitude and sonication time and second extraction yield dependence on sample to solvent ratio and sonication time. Based on the areas where the highest extraction yield appears, minimum values of parameters during process for maximum extraction effect have been calculated. Interdependence among process parameters as an independent variables and extraction yield as an dependent variable has been measured using 3D square graphs from which process parameters effects on extraction yield can be read. The rest of statystics is done using regular 2D scatter diagram and multiple regression.

4. RESULTS AND DISCUSSION

The main goal of this study was optimization of ultrasound assisted extraction (UAE) conditions, including extraction time (0.5-4 min), ultrasound amplitude (20 – 40%) and sample-to-solvent ratio (2.5:25, 5:25, 7.5:25), for the recovery of total carotenoids from orange peel. The variables and their values were selected on the basis of preliminary experiments. Total carotenoid yield was measured spectrophotometrically.

4.1. Solvent selection

Selection of appropriate solvent is one of the most critical factors for the efficient extraction of carotenoids. Conventionally, carotenoids are extracted using organic solvents.It is important to take into account thefunctional group (polarity) and chain length of the existing carotenoids, the sample matrix and its moisture content.

In order to select the most efficient organic solvent to extract carotenoids from orange peel five different solvents were analysed; ethanol, methanol, acetone, ethyl acetate and hexane. The amount of carotenoid extracted in five different solvents is presented in Table 5 and expressed in mg g^{-1}, showing how many miligrams of total carotenoid content are present in one gram of driedorange peel. From obtained results, it is clear that all solvents differ in their efficiency. The highest carotenoid yield (0.27 mg g^{-1}) was obtained when carotenoids were extracted with ethyl acetate. Extraction with methanol showed higher carotenoid yield in comparison with hexane and acetone, but lower than with ethyl acetate. Samples extracted with ethanol had the lowest yield of total carotenoids (0.20 mg g^{-1}).

Table 5. Total carotenoids content (mg g^{-1}) of orange citrus peel using different solvents

Solvent	Total carotenoids (mg g^{-1})	Standard deviation
Acetone	0.23045	0.032760
Ethanol	0.20480	0.021543
Ethyl acetate	0.27573	0.019065
Hexane	0.23033	0.006337
Methanol	0.26968	0.003238

Values are means ± SD (*n* = 3).

Solvent selection for ultrasound assisted extraction is driven by the solubility of the target compounds but also by physical paramaters such as viscosity, surface tension and vapor pressure of the solvent. These physical parameters affect acoustic cavitation phenomenom and cavitation threshold which cause direct influence on the efficiency of extraction. (Chemat et al., 2017). Due to presence of both types of carotenoids in orange peel, polar xanthophylles and non polar carotenes, solvent for carotenoids extraction should not have too high or to low polarity. Polarity index is a measure of the relative polarity of a solvent. Polarity index for ethyl acetate (4.4) is lower than for ethanol (5.2) and higher than for hexane (0). In addition, in order to get more efficient extraction, it should be looked for the extraction solvent with low viscosity, low density and high diffusivity. Ethyl acetate exhibit medium viscosity (0.45 cP),lower than ethanol (1.2 cP) and higher than acetone (0,32 cP). Those characteristics enable solvent to diffuse into the pores of plant material (Londono-Londero et al., 2010). Furthermore, Singanuseng et al. (2014) point out that lower viscosity helps solvent to achieve phenomenom of acoustic cavitation so ultrasonic waves can easier exceed the intermolecular forces of the liquid. For instance, the lowest extraction yield was with ethanol maybe due to its too high polarity and higher viscosity compared to other solvents as well. On the other side, hexane is typical non polar organic solvent and that could be reason for the lower extraction yield because xanthopylles are soluble in polar solvents. Altough, Ghazi (1999) found out that mixture of hexane and acetone (1/1 v/v) yielded the highest amount of β-carotene from orange peels possibly because β-carotene as a fat-soluble pigment is soluble in non-polar organic solvents such as hexane.Acetone's polarity is similar to ethanol's and methanol's but perhaps higher vapor pressure could reduce extraction yield, thus could result in less violence and intensity of bubble collapse (Singanuseng et al., 2014). However, since carotenoids naturally occur inside plant cells and are surrounded by an aqeus protoplasmic medium, acetone might be useful due to its ability to bind water and force carotenoids out of the solution (Ghazi, 1999). Methanol was very good solvent even though it is very polar compound. In conducted assays, it was determined that ethyl acetate is the most suitable solvent for ultrasound assisted extraction of carotenoids from orange peel and it was not suprise because ethyl acetate has desirable properites; medium polarity, higher boiling point, lower viscosity and average surface tension. Sun et al. (2011) also reported that ethyl acetate was the most suitable for the extraction of all-trans-β-carotenes in the mandarine peel, but on the other side they pointed out that ethanol could be a good choice, due to its lack of toxicity which is not the case with ethyl acetate. In another study, Yan et al. (2015) found out that among five different solvents, the highest carotenoid content yielded out with the use of petroleum ether/acetone (V/V = 1/1), but it should be mentioned that carotenoids were extracted from rapeseed meal.

4.2. Optimal combination of extraction time and amplitude

For the inital sample weight of 2.5 grams, respectively sample to solvent ratio 2.5:25, results of the extraction efficiency based on different parameter combinations are shown in Figure 17. It can be noticed the highest extraction peak has been achieved with maximum amplitude of 40 % and maximum sonication time of 4 minutes. It seems like a logicall result primary due to low sample to solvent ratio (inital sample weight) and consequently avoidance of potential undesirable effects which might occur at bigger inital sample weights due to high saturation and heat generation.

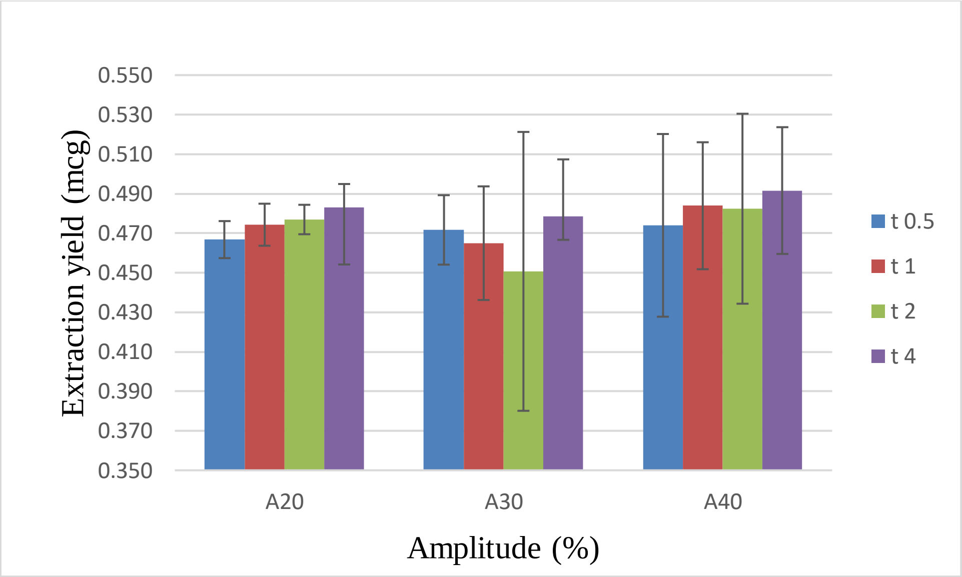

Figure 17. Effect of amplitude and sonication time on extraction yield for inital sample weight of 2.5 g

For the inital sample weight of 5 grams, respectively sample to solvent ratio 5:25, results of the extraction efficiency based on different parameter combinations are shown in Figure 18. It can be noticed the highest extraction peak has been achieved with the combination, amplitude of 30 % and maximum sonication time of 4 minutes. Generally for every of three different amplitude extraction yield increases with the sonication time. However it is not the case with amplitude and the highest yields are obtained when amplitude is 30 %, moreover at higher amplitude, efficiency of extraction drops. Higher levels of amplitude might change ultrasound tranducer efficiency, and instead of cavitation, agitation might occur and potentially cause impairment of ultrasound transmission through sample.

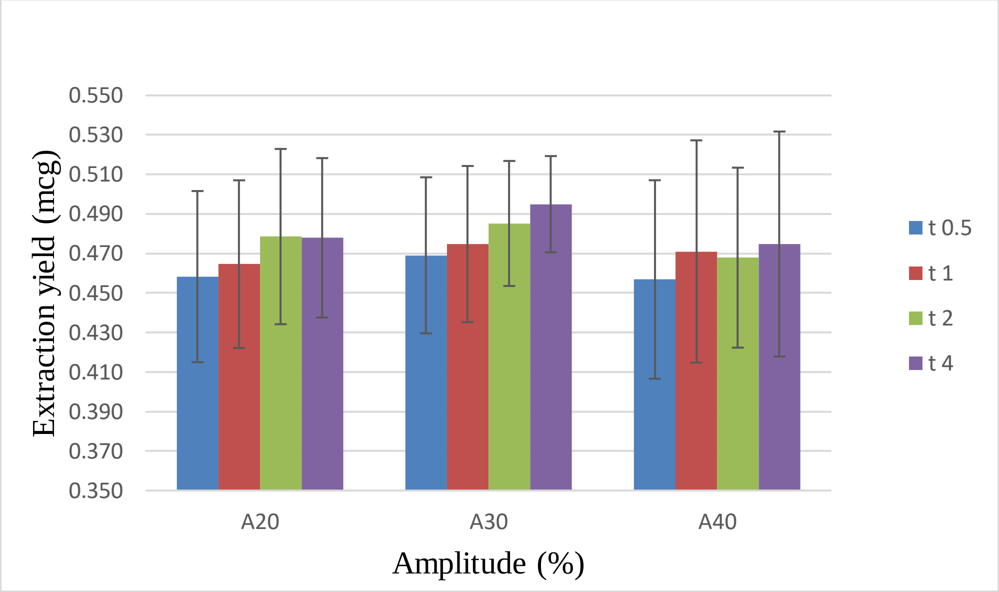

Figure 18. Effect of amplitude + sonication time on extraction yield for inital sample weight of 5 g

For the inital sample weight of 7.5 grams, respectively sample to solvent ratio 7.5:25, results of the extraction efficiency based on different parameter combinations are shown in Figure 19. It can be noticed the highest extraction peak has been achieved with the combination, amplitude of 30 % and maximum sonication time of 4 minute. Furthermore, the highest yields in average are obtained when amplitude is 30 %, while raising with the time. The solvent saturation might be a limiting extraction due to the higher inital sample mass. It could lead to a delay in the extraction rate, which could subsequently resulted in carotenoid content reduction.

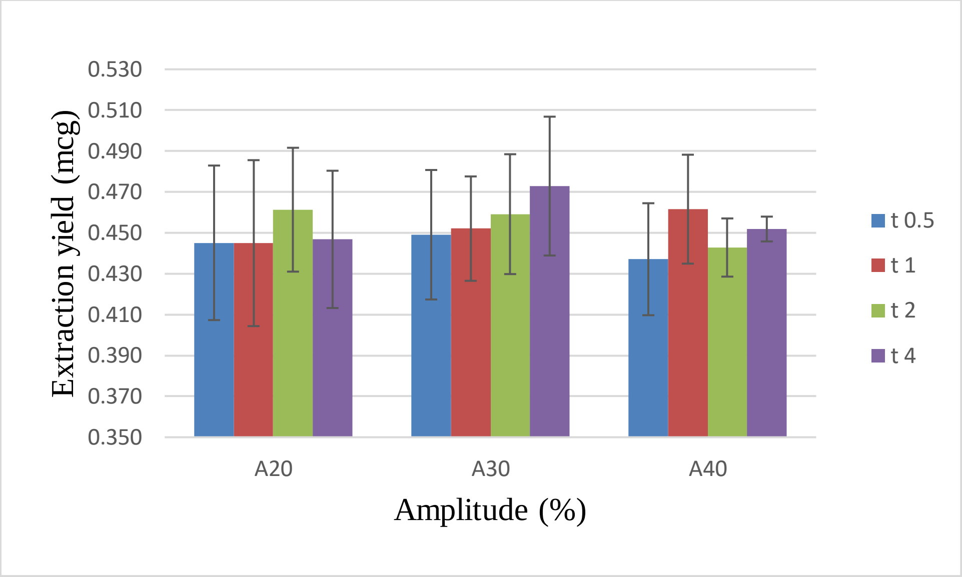

Figure 19. Effect of amplitude sonication time on extraction yield for inital sample weight of 7.5 g

4.3. Optimal combination of amplitude and sonication time

Based on the graph which is shown at Figure 20 it can be noticed, the highest extraction yield is achieved at maximum amplitude of 40 %, whereby extraction yield increases with the sonication time and the maximum value occurs when process goes under sonication time of 4 minutes.

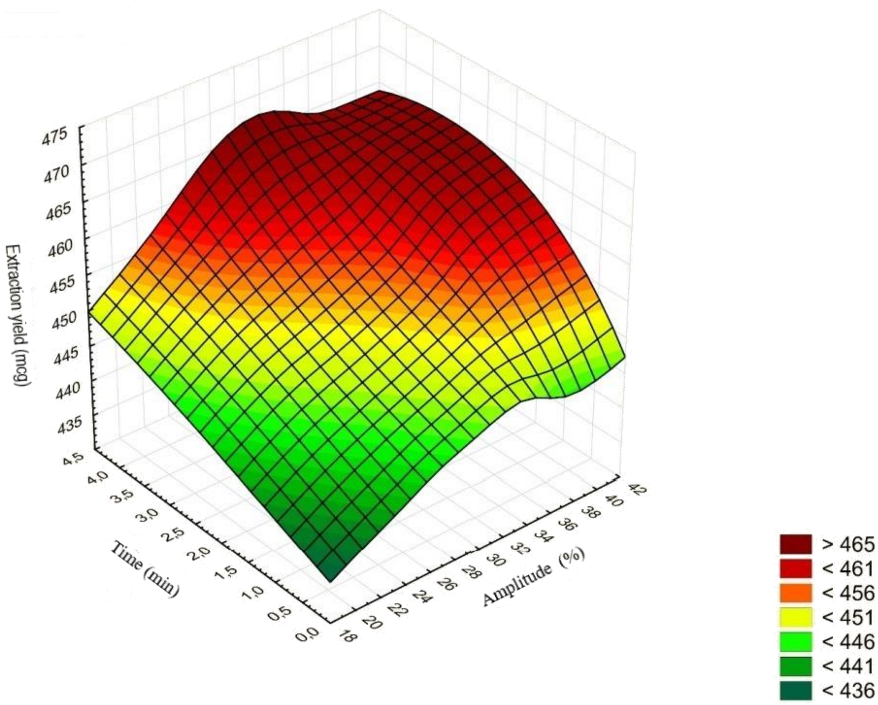

Figure 20. Extraction yield (mcg) dependence on different combinations between parameters, amplitude (%) and sonication time (min)

Depending on whether goal is to minimize ultrasound amplitude or reduce sonication time, different combinations of optimal parameters could be done. Hence, in Figure 21 results of different combinations are presented. In order to influence on sample material with the as low as possible amplitude, optimum parameters for amplitude are 28 – 30 % with sonication time of 4 minutes. On the other hand, when goal is to minimize sonication time, samples should be treated with maximum amplitude (36 – 40 %) under 2.4 minutes of sonication.Every following increase in sonication time under maximum amplitude has not brought higher extraction yield which suggest that mutualy high amplitude and long sonication time excessively increase the temperature, consequently produceing heat generation which possible impairs extraction process. Temperature affects the yield of the extraction process by changing the solubility of the solute in the solvent used for extraction and also by way of altering the properties of solvent such as viscosity and density, which have a significant effect on the cavitational activity (Purohit and Gogate, 2015). Interestingly, temperature has two opposite effects, since it enhances mass transfer during extraction but also promotes higher degradation rates. From the cavitation effect point of view, increasing temperature had a negative effect because the cavitation intensity decreases with increasing temperature. On the other side from the thermal effect point of view, the increase in temperature had a positive influence because of the same reason as under carotenoid extraction (Sun et al., 2011).

Multiple regression which has been implemented has shown extraction yield dependence on level of amplitude with p = 0.000123 and furthermore, extraction yield dependence on sonication time with p = 0.002953.

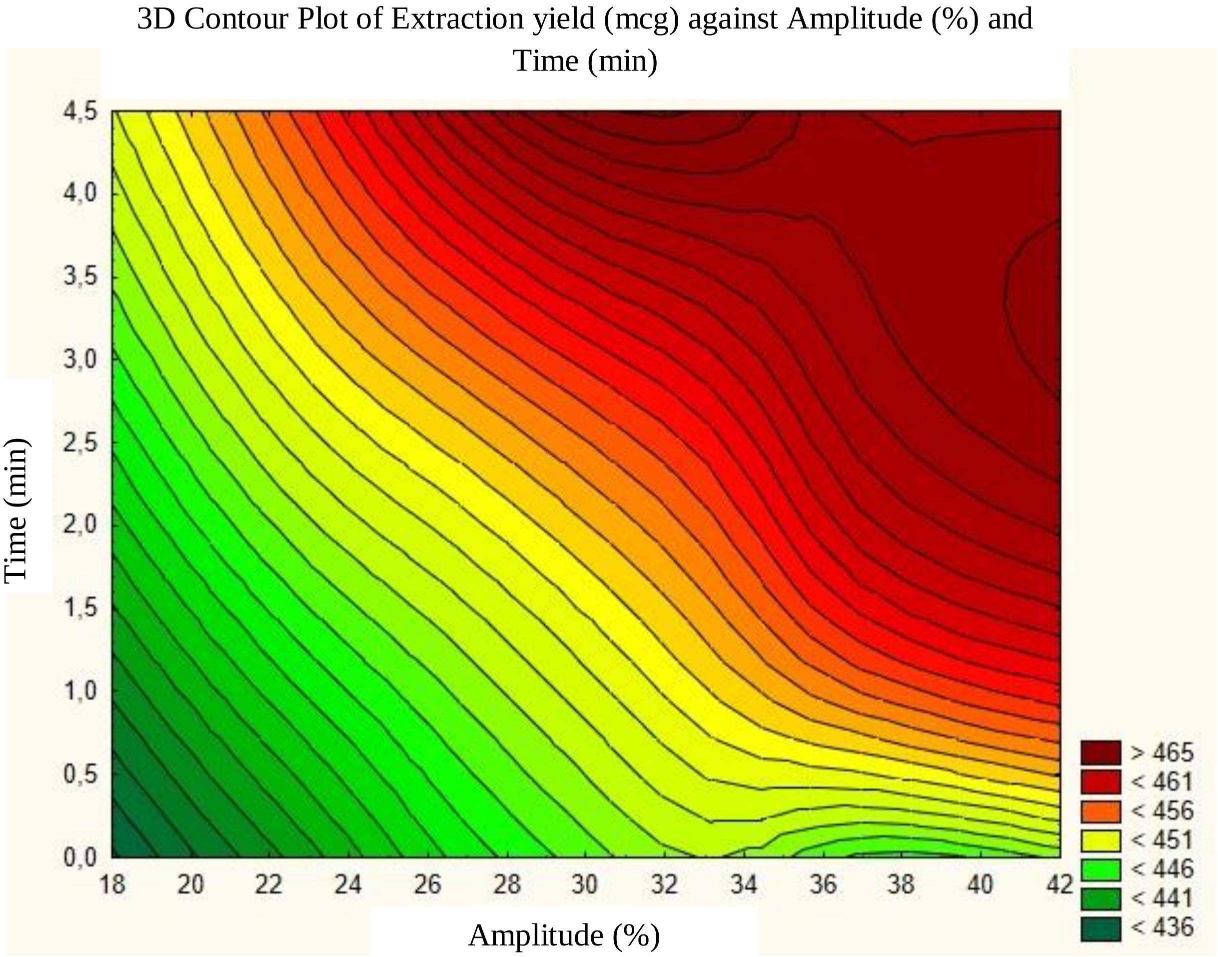

Figure 21. 3D contour plot of extraction yield (mcg) dependence on different combinations of amplitude (%) and sonication time (min)

4.4. Optimal combination of amplitude and sample to solvent ratio (inital sample weight)

With the increase of initial sample mass, logically extraction yields are higher. In this case, dependence of extraction yield on power, respectively amplitude of ultrasound. The highest yield are obtained at maximum amplitudes. Based on the Figure 22, it can be concluded that amplitudes above 30 % are not more efficient in extraction and as such are not necessary for use. Considering that the solvent volume is constant 25 mL, it is clear that inital dry sample mass affects the efficiency of UAE. If sample concentration differs, consequently rate of saturation inside falcon tube differs and it influences basic physical conditions during UAE.

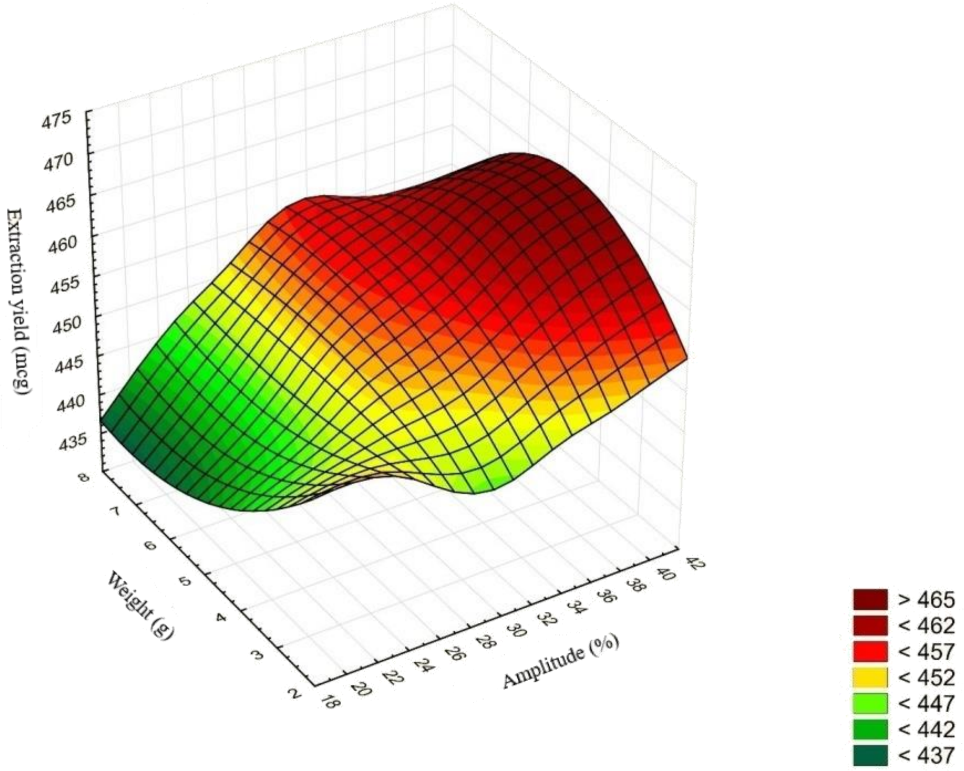

Figure 22. Extraction yield (mcg) dependence on different combinations of amplitude (%) and inital sample weight (g)

Dahmoune et al. (2014) had confirmed that interaction between amplitude and solvent was significant therefore affecting the extraction yield. Even tough they had been extracting phenols from the *citrus sinensis L.* peels, they underlined that this interactions influences its release from different matrix which can modify equilibrium and mass transfer conditions in the solvent extraction and affect rupture of cell wall.

4.5. Amplitude effect on extraction yield

Figure 23 shows how amplitude power affects rate of extraction yield. First increase in amplitude power from 20 % to 30 % is followed with almost linear increase in extraction yield. Second increase from 30 % to 40 % is still followed with increase in extraction yield but not in linear progression anymore and at slower rate of raise. Santos et al. (2009) assert even though increasing the amplitude can increase ultrasound intensity, on the other side high amplitudes can lead to rapid deterioration of the ultrasonic transducer which can result in liquid agitation instead of cavitation and consequently in poor transmission of the ultrasound through the liquid media.

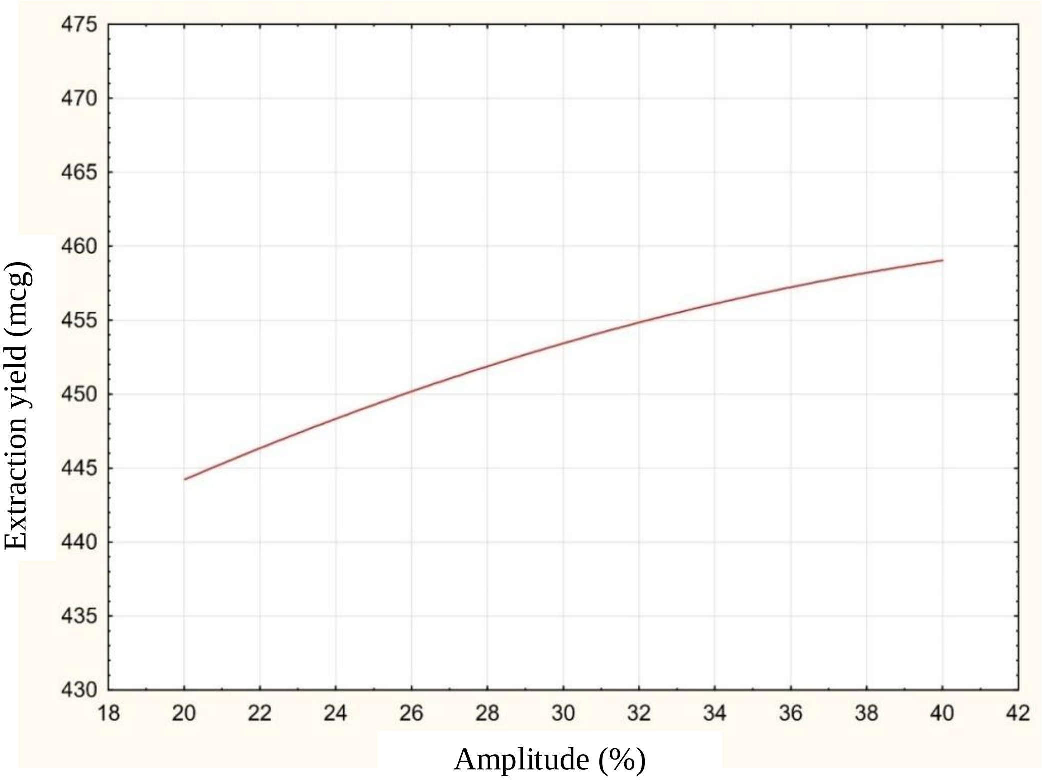

Figure 23. Influence of the amplitude (%) on extraction yield (mcg)

However, there are two ways to increase physicichemical effects of ultrasound, to increase the number of cavitating bubbles or to increase the power of bubble implosion. At higher vibration amplitudes, the effective size of the liquid zone undergoing cavitation expands and the range of bubble size underoing cavitation increases as well. (Luengo et al., 2014). For instance, Luengo et al. (2014) indicated that the maximum carotenoid extraction yield from tomato was obtained by applciation of vibration amplitude of 94 µm and external pressure of 50 kPa.

Figure 24 shows $p = 0.00059 < 0.05$, hence considering that data, extraction yield depend on applied amplitude.

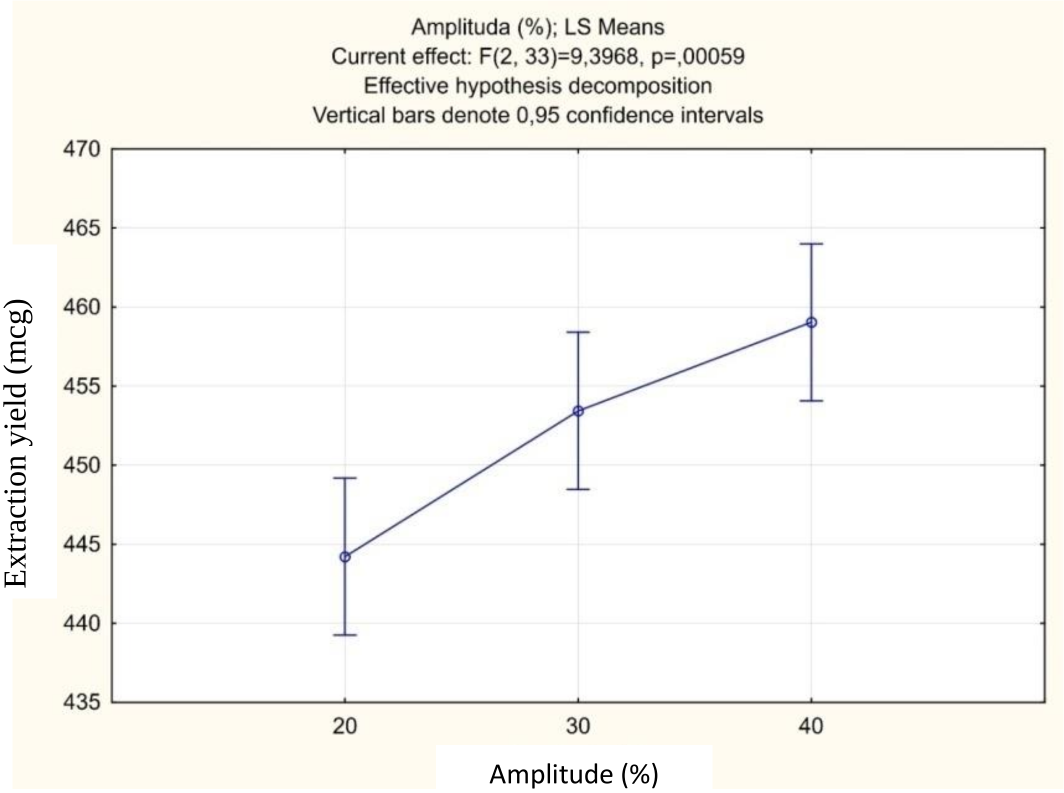

Figure 24. One way (single factor) ANOVA for extraction yield (mcg) dependence on amplitude power (%)

According to post-hocTukey HSD test in table 6. it can be noticed, the highest extraction yield is achieved when amplitude is 40 %.

Table 6. Extract yield (mcg) dependence on amplitude power (%)

	Tukey HSD test; variable extract yield (mcg)			
Cell No.	Amplitude (%)	{1} 444,21	{2} 453,43	{3} 459,04

1	20		0,030685	0,000521
2	30	0,030685		0,250523
3	40	0,000521	0,250523	

4.6. Effect of sonication time on extraction yield

Observing the results shown in Figure 25. it may be assumed that sonication time drastically affects the final extraction yield, especially in first two minutes of ultrasound process when yield linearly increases with the time. The highest yield are achieved with the longest sonication time of 4 minutes. Prokopov et al. (2017) concluded it is due to the large carotenoid concentration gradient between the solvent and the plant cells at the beggining of the extraction. This gradient decreased with the increase in the extraction time due to the increased mass transfer caused by ultrasonic treatment and consequently the extraction of carotenoids from the inside of the cell gradually become more difficult. Also, results show that the extraction yield increases with the sonication time and in this experiments the longest time of 4 minutes obtained the highest yield. Chuyen et al. (2018) found out that carotenoid extraction yield from Gac peel gradually increased with the extraction time independently of ultrasound power. But to be noticed, they used longer period of sonication and for example found out the highest yield peak at 60 min for ultrasound power of 250 W.

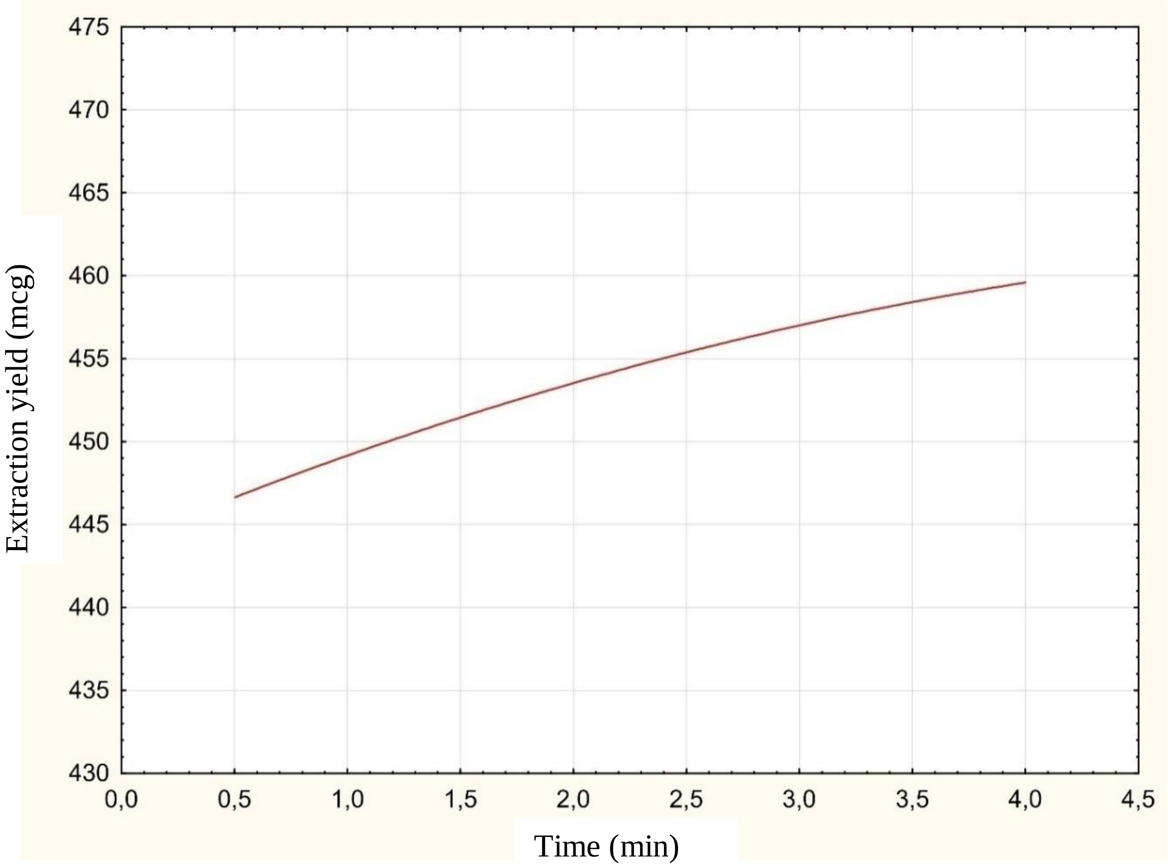

Figure 25. Influence of sonication time (min) on Extraction yield (mcg)

Prokopov et al. (2017) found out the highest total carotenoid content from tomato cultures after 5 and 10 minutes of sonification. Interestingly Garcia-Castello et al., (2015) found out extraction yield at very short times similar to that obtained at longer times in process of flavonoids extraction from grapefruit. Their findings shows almost the same yield value at 3 min to that at 32 min, however they did not find out sonication time as a significant variable.

Many other studies were also indicated that the application of ultrasound in the extraction of carotenoids can reduce the extraction time compared to the conventional extraction methods. For example the extraction yield of lutein from egg yolk using ultrasound for 10 min was four times higher that the yield obtained from the conventional extraction with hexane for 20 min (Yue et al. 2006). The extraction time for recovering B-carotene from carrots was also shown to be reduced three times using ultrasound assisted extraction (Li et al. 2013).

It can be seen with $p = 0.002279 < 0.05$ extraction yield depend on sonication time.

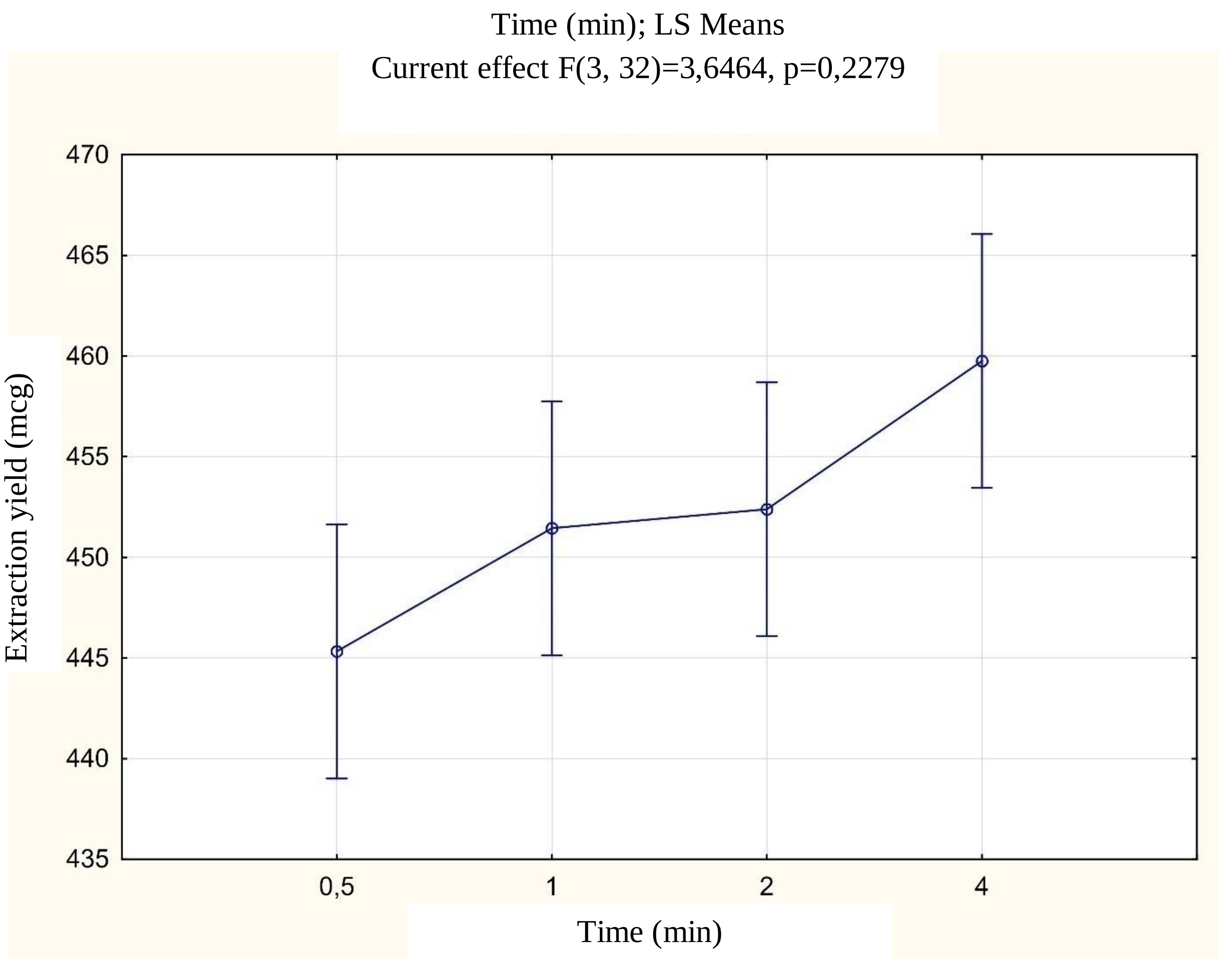

Figure 26. One way (single factor) ANOVA for extraction yield (mcg) dependence on sonication time (min)

4.7. Effect of sample to solvent ratio and sonication time on extraction yield

In Figure 27, it can be seen the highest extraction yield is obtained when sonication time is 4 minutes and when inital sample mass is between 5 and 7.5 g, respectively when sample to solvent ratio is between 5:25 and 7.5:25. In order to get as high as possible yield from as low as possible inital sample weight, optimal paramters are noted at sonication time of 2.6 min and inital sample weight of 4.8 g. Ultrasound can improve extraction yield due to many different physical phenomenoma which causes, such as fragmentation, erosion, sonocapillary effect, sonoporation, detexturation (Chemat et al., 2017). However, if liquid is oversaturated all this phenonomenoms are possibly not carried out like as at optimal saturation level, furthermore mass transfer might be impaired what can reduce possibility bubbles to reach cavitation threshold which is essential for achieving ultrasound effect.

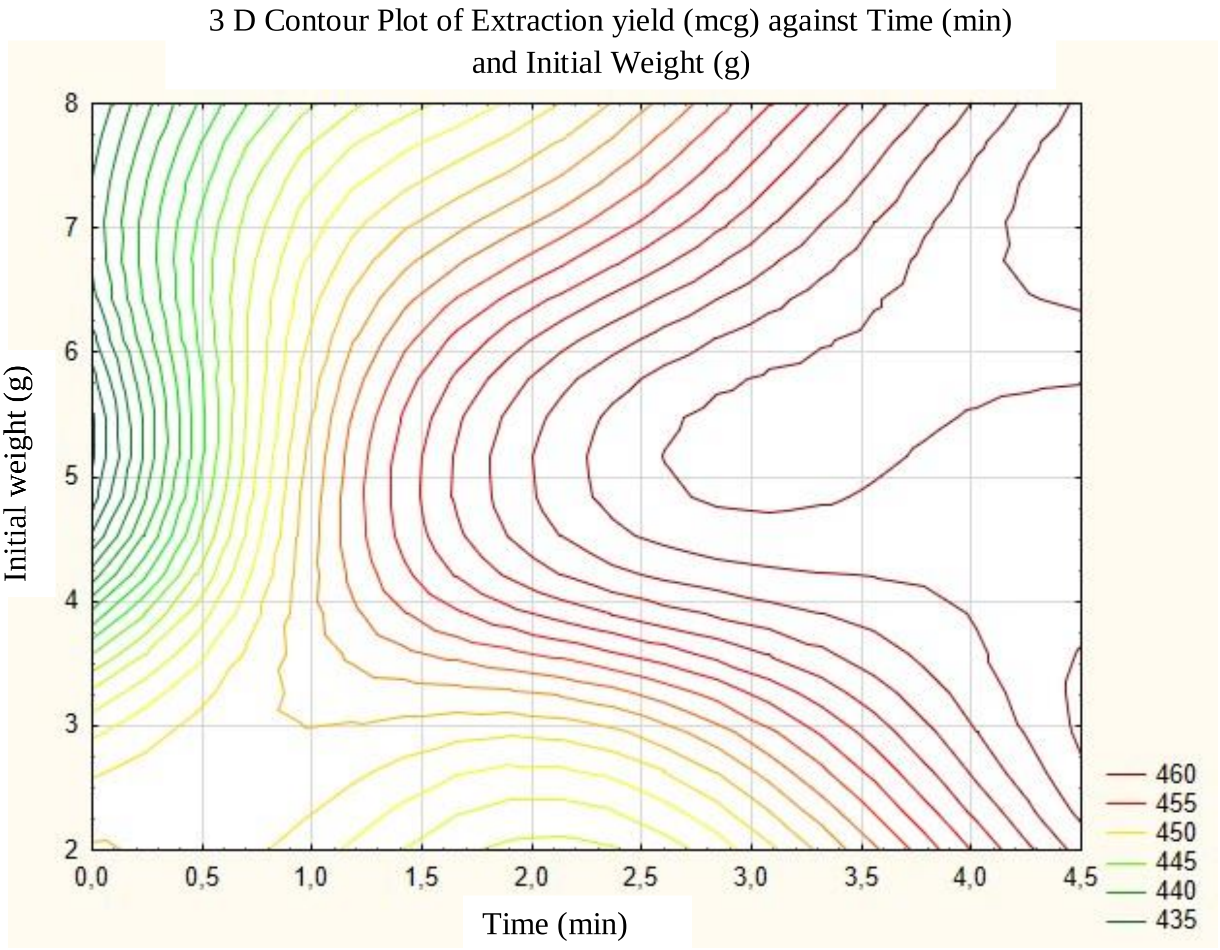

Figure 27. 3DContour plot of extraction yield (mcg) dependence on sonication time (min) and initial sample weight (g)

Effect of higher inital sample weight were not measured but there might be a counter effect regarding too high sample concentration might affect physicall properties of sample during ultrasound treatment, for example viscosity, diffusivity, vapor pressure and density and changes in those properties can generally reduce efficiency of extraction.

5. CONCLUSIONS

Based on results and discussion presented in this Thesis, the following conclusions can be drawn:

1. Assays has identified, ethyl acetate is the most suitable solvent for ultrasound assisted extraction of carotenoids due to its desirable properties; medium poarity, higher boiling point, lower viscosity and average surface tension.
2. From the executed different combinations of amplitude power and sonication time throughout assays, mutual effect of maximum amplitude of 40 % and maximum sonication time of 4 minutes has been deretmined as an optimal paramters in terms of the highest extraction yield.
3. In terms of power economy, amplitude of 28 – 30 % is the lowest possible which acomplishes significant influence on sample with time of 4 minutes.
4. In terms of time sparing, in combination with maximum amplitude (36 – 40 %), it is enough to threat samples under 2.4 minutes.
5. In order to obtain as high as possible yield from as low as possible inital sample weight sonication time of 2.6 min and initial sample weight of 4.8 g (4.8:25 sample to solvent ratio) should be used.
6. With the increase of sample to solution ratio (initial sample weight), extraction yields raise but regarding amplitude power, amplitudes above 30 % are not more efficient in extraction and as such are not necessary for use.

6. LITERATURE

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STATEMENT OF ORIGINALITY

This is to certify, that the intellectual content of this thesis is the product of my own independent and original work and that all the sources used in preparing this thesis have been duly acknowledged.

Roko Marović