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MAJA ČREŠNIK

MICROENCAPSULATION OF LIQUID FORMULATIONS WITH ANTIOXIDANTS BY VIBRATION NOZZLE METHOD

MIKROKAPSULIRANJE TEKOČIH FORMULACIJ Z ANTIOKSIDANTI Z METODO VIBRIRAJOČE MEMBRANE

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Diplomsko nalogo – smer Kozmetologija in njen praktičen del sem opravljala na Faculté de pharmacie de Marseille, Katedri za galensko farmacijo, biofarmacijo, farmacevtsko tehnologijo in industrijsko kozmetologijo, pod mentorstvom prof. dr. Philippe Piccerelle in somentorstvom prof. dr. Christophe Sauzet, teoretični del naloge pa na Fakulteti za farmacijo, Katedri za farmacevtsko tehnologijo, pod mentorstvom prof. dr. Mirjane Gašperlin.

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Maja Črešnik

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ABSTRACT

Microencapsulation is a process by which very small droplets, solid particles or air bubbles (the core) are coated with continuous film of polymer, lipid or other suitable material (the shell) to produce microcapsules. They can be manufactured from a wide range of natural or synthetic materials. In the field of cosmetic science there are many ingredients, which are desired to be microencapsulated. These ingredients are UV-filters, antioxidants, pigments, botanical extracts, essentials oils, natural oils, fragrances, etc. The main reasons for microencapsulation are protection against UV-light, heat, chemical reactions (such as oxidation) or to enhance solubility, achieve controlled and targeted release, etc.

In diploma thesis we focused on microencapsulation of liquid formulations with antioxidants by vibration nozzle method with concentric nozzle system. Microcapsules were composed of a core and a shell. For shell material we used natural polymer sodium alginate and for core material we prepared three different formulations with antioxidants. We used two lipophilic antioxidants, resveratrol and beta-carotene, which were dissolved in almond oil. As the third formulation for hydrophilic type of antioxidants (e.g., niacinamide) we prepared water in oil emulsion (W/O). For satisfactory preparation of microcapsules, we determined optimal process parameters, such as electrode, frequency, flow rate, pressure, etc., which were indicated by visualization of bead formation in the stroboscope of encapsulator. After successful preparation we determined properties and quality of liquid core microcapsules. Using light microscopy we determined morphology, thickness of shell and rate of encapsulation. We found the produced microcapsules to be mononuclear and most of them in spherical form. Thickness of shells was fairly thin and degree of encapsulation was around 60%. Thickness of shell and degree of encapsulation significantly affect the release of active ingredients from microcapsules. Microcapsules size distribution was measured by laser diffraction. Results show that size distribution is not uniform for all microcapsules; however it approximately follows the log-normal distribution. One of the most important properties of microcapsules is encapsulation efficiency, which was measured by UV-Vis spectrophotometer. According to measured absorbance of beta carotene in almond oil solution after microencapsulation, we determinated that encapsulation efficiency was 79 %. The result is satisfactory; however encapsulation efficiency in ideal conditions can reach up to 90 %.

We concluded that vibration nozzle method represents suitable and effective means for protection of sensitive cosmetic active ingredients such as antioxidants.

POVZETEK

Mikrokapsuliranje je proces, s katerim se posamezni delci ali kapljice trdnega ali tekočega materiala (jedro) obdajo s kontinuiranim filmom polimernega materiala (ovojnica), pri čemer nastanejo kapsule v mikrometrskem območju, imenovane mikrokapsule. Za proizvodnjo le teh imamo na voljo veliko naravnih ali sinteznih materialov. Na področju kozmetologije je veliko sestavin, ki morajo biti za učinkovito delovanje mikrokapsulirane. Take sestavine so UV-filtri, antioksidanti, pigmenti, rastlinski ekstrakti, eterična olja, naravna olja, dišave, itd. Glavni razlogi za mikrokapsuliranje so zaščita snovi pred UV-žarki, toploto, kemijskimi reakcijami (zlasti oksidacijo), uporabljajo pa jo tudi za izboljšanje topnosti ter doseganje nadzorovanega ali ciljanega sproščanja, itd.

V diplomski nalogi smo se osredotočili na mikrokapsuliranje tekočih formulacij z antioksidanti z metodo vibrirajoče membrane s koncentričnim sistemom šob. Mikrokapsule so bile sestavljene iz jedra in ovojnice. Za ovojnico smo uporabili naravni polimer natrijev alginat, za jedro pa smo pripravili tri različne formulacije z antioksidanti. Uporabili smo dva lipofilna antioksidanta, resveratrol in beta-karoten, ki sta bila raztopljena v mandljevem olju. Kot tretjo formulacijo, model hidrofilnega tipa antioksidantov (npr. niacinamid), smo pripravili emulzijo tipa voda v olju (V/O). Za uspešno pripravo mikrokapsul smo morali najprej proučiti optimalne procesne parametre kot so elektroda, frekvenca, pretok tekočin, tlak, itd. Ti parametri so bili določeni vizualno glede na nastajanje kapljic, ki so bile osvetljene z lučjo enkapsulatorja. Po uspešni pripravi smo določili lastnosti in kakovost mikrokapsul. S svetlobnim mikroskopom smo raziskali morfološke lastnosti, debelino ovojnice in stopnjo mikrokapsuliranja. Ugotovili smo, da so pripravljene mikrokapsule mono-jedrne in da je večina sferičnih. Debelina ovojnice je bila dokaj tanka, stopnja enkapsuliranja pa je bila okoli 60 %. Debelina ovojnice in stopnja kapsuliranja pomembno vplivata na sproščanje aktivnih sestavin iz mikrokapsul. Porazdelitev velikosti mikrokapsul smo izmerili s pomočjo laserske difrakcije. Rezultati so pokazali, da porazdelitev velikosti ni enotna za vse mikrokapsule, vendar mikrokapsule še sledijo log-normalni porazdelitvi velikosti. Najpomembnejša lastnost mikrokapsul je

učinkovitost mikrokapsuliranja, ki smo jo izmerili s pomočjo UV-Vis spektrofotometra. Glede na izmerjeno absorbanco spojine beta-karotena v mandljevem olju po mikrokapsuliranju, smo določili, da je učinkovitost kapsuliranja 79%. Rezultat je zadovoljiv, čeprav lahko pod idealnimi pogoji dosežemo do 90% učinkovitost.

Zaključili smo, da je z metodo vibrirajoče membrane možno učinkovito zaščititi občutljive kozmetično aktivne učinkovine kot so antioksidanti.

LIST OF ABBREVIATION

AO	Antioxidant
MC	Microcapsule
RSV	Resveratrol
VN	Vibration nozzle

NOMENCLATURE

d_d – droplet diameter (µm)

d_n – nozzle diameter (µm)

σ – surface tension (N/m)

g – acceleration due to gravity (m/s^2)

ρ – fluid density (kg/m^3)

λ_{opt} – optimal wavelength (µm)

λ – wavelength of perturbation (µm)

v_j – jet velocity (m/s)

f – vibrational frequency (1/s)

V' – flow rate (mL/min)

η – fluid viscosity (kg/ms)

d_m – mean diameter of microcapsule (µm)

d_c – mean diameter of microcapsule core (µm)

M_m – mean size of microcapsule membrane (µm)

V_C – volume of microcapsule core

V_{MK} – volume of microcapsule

C_0 - concentration of beta-carotene in almond oil before microencapsulation

C_1 - concentration of beta-carotene in almond oil after microencapsulation

1. INTRODUCTION

1.1. NOVEL COSMETICS DELIVERY SYSTEMS

Since 1980s, cosmetic industry has increased scientific research in the development of highly effective cosmetics products. Reasons for such research were consumers' needs for ingredients that more considerably beautify and also treat the skin (1).

The main concern in cosmetics is to reach cutaneous cells while limiting the passage into the blood circulation. Therefore, special delivery systems have been developed in personal care formulations (2). Cosmetic industry is currently growing immensely and its main objective is to pack the active ingredients into an appropriate delivery system. Delivery systems promote ingredients to be efficiently delivered to the target area (Figure 1), while preserving their biological activity or chemical nature (3). Delivering agents to the targeted site requires the right concentration of in the formulation to reach optimal release and required distribution of active ingredient between the vehicle and target site (2).

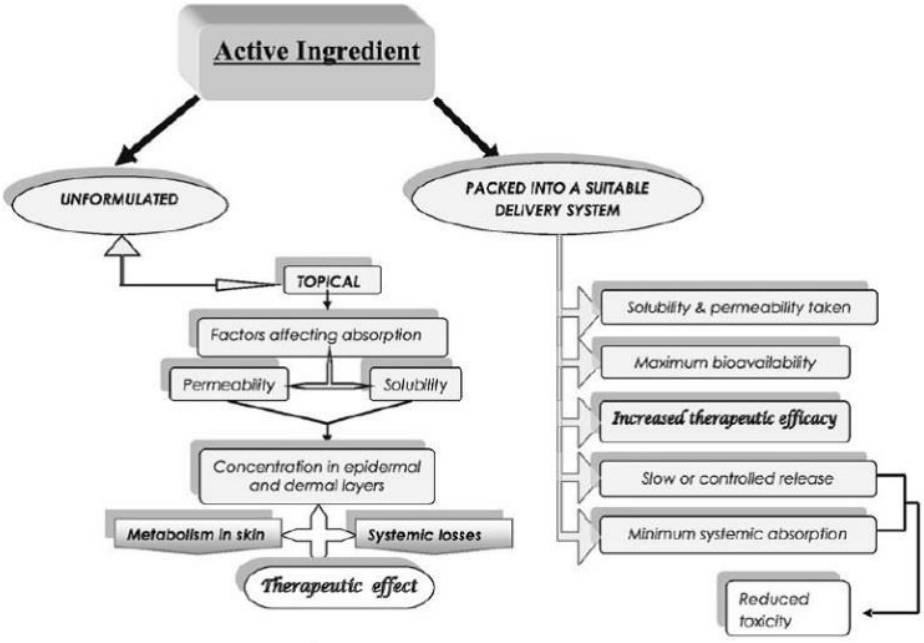


Figure 1: Benefits of using suitable delivery system in relation to topical administration of active substances (1).

1.2. OXIDATIVE SRESS

Skin is the largest human organ and because of the high content of biological targets, it is very sensitive to oxidative stress (1, 3). Well known initiators of free radicals and consequently of oxidative stress are metabolism, ultraviolet light and environmental pollutants (1, 3, 4). Free radicals are molecules with unpaired electrons, which make radicals highly reactive and may result in injury to surrounding molecules and tissues. Oxidative stress is present in living organisms, because of the increased amount of reactive oxygen species (ROS). It is a disturbance in equilibrium of produced free radicals and antioxidant protection (3). The most considerable injury by free radicals is to DNA and biomembranes (1). Increasing the amount of free radicals can trigger photoageing, wrinkling, drying, elastosis and hyperpigmentation of the skin.

1.2.1. ANTIOXIDANTS

Skin is equipped with nonenzymatic and enzymatic antioxidant systems; including super oxide dismutase (SOD), glutathione peroxidase, catalase, ascorbate, tocopherols, etc., to prevent oxidative damage (3). It is scientifically proven, that topically applied antioxidants can interrupt the chain reactions by removing the intermediates of free radicals and inhibit other oxidation reactions by being oxidized themselves. This can defend the skin against the environmental stress caused by free radicals (5). It is known that AOs, which are applied on the skin, are able to penetrate into the upper layers of epidermis (6). Therefore, many cosmetic products contain AOs as the active ingredients (5). However, AOs remain in the stratum corneum for a short time, as they are of quickly lost due to desquamation, textile contact and washing (6).

There are two major advantages in applying a topical AOs formulation on the skin. Firstly, the skin reaches far higher levels of each AO, as to taking these AOs orally. Secondly, topical application supports the skin with a supply of AOs, which cannot be scrubbed or washed off. It is a protection that remains in the skin even many days after application. Topically applied AOs are proven to prevent skin damage caused by oxidative stress, if they are used in time (3).

The main problem of AO molecules is that several of them can degrade in the presence of oxygen, light and water. It is not easy to formulate an acceptable and stable

AO formulation for cosmetic use, because of their instability. Therefore, delivery systems that improve stability, solubility and permeability of AOs are required (3).

The general accepted criteria for all active cosmetic ingredients are: stability during production, storage and use, and demonstration of the desirable effects at the targeted site after application (1).

1.3. MICROENCAPSULATION

Microencapsulation is a process by which very small droplets, solid particles or air bubbles (the core) are coated with continuous film of polymer, lipid or other suitable material (the shell) to produce capsules in the micrometer range, known as microcapsules (MCs) (7). They can be manufactured from a wide range of natural or synthetic materials (8).

Microencapsulation was developed by mimicking the processes in the natural processes. Nature protects materials from the environment with a shell; most basic examples are bird eggs with in its content (9).

MCs can take many structural forms and are proven to have many exploitable characteristics for application in many different processes. The concept of encapsulating materials with a defined membrane dates back to the 1930s in the food industry. In the following years, microencapsulation technology was applied successfully to cosmetic, pharmaceutical, chemical, and food industries, as well as agriculture and printing (8).

In the field of cosmetic science there are many ingredients, which are desired to be microencapsulated. These ingredients are UV-filters, antioxidants, pigments, botanical extracts, essentials oils, natural oils, fragrances, etc. Microencapsulated ingredients are included in a various cosmetic products such as shower and bath gels, colour cosmetics (lipstick, make-up), wrinkle creams, sunscreens and tanning creams, aromatherapy products, soaps, exfoliants, hand creams and body lotions (10) (Figure 2).

Figure 2: Microcapsules included in various creams (2).

1.3.1. MATERIALS

Preparation of MCs requires basic understanding of the nature of the core and shell materials, stability and release properties of the shell materials and the microencapsulating methods (11).

Core material:

- Solid or liquid
- Liquid core material may be dispersed or dissolved
- Composition of core material: drug or active substance, additives as diluents, stabilizers and release rate enhancers (7).

Shell material:

- Inert substance that coats the core with desired thickness
- Compatibility with the core material
- Stabilization of the core material
- Inert towards active ingredients
- Controlled release under certain conditions
- The shell can be flexible, fragile, thin, hard, etc.
- Cheaply and abundantly available
- Composition of shell: inert polymer, plasticizer, colouring agent (7) (Examples are presented in Table I).

The selection of a specific shell material can be made from a long list of materials, based on the following questions to be considered by the researcher (11):

- What is the specific purpose of product requirement – stabilization, decreased instability, release properties, environmental conditions, etc.?
- What shell material will satisfy the aims and requirements?
- Which microencapsulating method is the best appropriate to accomplish the coated product aims?

The selection of the suitable shell material defines physical and chemical characteristics of MCs (11).

Table I: Commonly used shell materials for microencapsulation.

Water soluble resins	Water insoluble resins	Waxes and lipids	Gastro-resistant resins
Gelatin	EC – Ethyl cellulose	Paraffin	Shellac
Gum Arabic	Polyethylene	Carnauba	Cellulose acetate phthalate
Alginates	Polymethacrylate	Beeswax	Zein
Starch	Polyamide (Nylon)	Stearic acid	
PVP - Polyvinylpyrrolidione	Cellulose nitrate	Stearyl alcohol	
CMC - Carboxymethylcellulose	Silicones	Glyceryl stearates	
MC - Methyl cellulose		Spermaceti	
HEC - Hydroxyethylcellulose			
Arabinogalactan			
Polyvinyl alcohol			
Polyacrylic acid			

Liquid core capsules

Recently, the availability of capsules having a liquid core, show a common interest from cosmetic industry:

- hydrogel capsules with an organic core can be used as controlled release tool for flavours and fragrances,
- liquid core capsules enable in-situ extraction of organic ingredients in a fermentation process (12).

1.3.2. MORPHOLOGY OF MICROCAPSULES

The core material and the deposition process of the shell material on the core are two major properties that have impact on the morphology of MC. There are three different types of MCs (Figure 3):

I. Mononuclear (core-shell) MCs consists of the shell around a single core.
II. Polynuclear capsules have several cores enclosed inside the shell.
III. Matrix MCs in which the core material is homogeneously distributed inside the shell material, are known as microspheres (7).

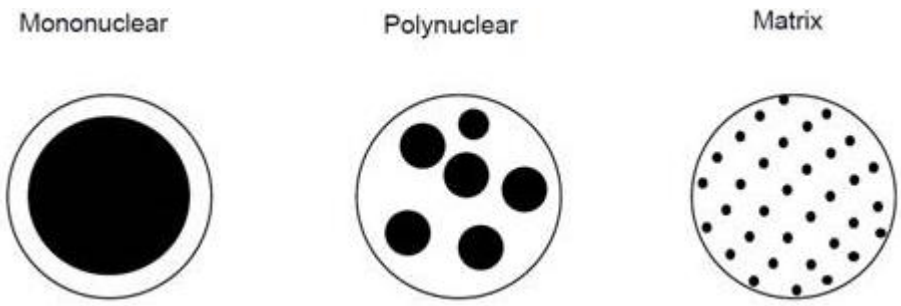


Figure 3: Morphology of microcapsules (3).

1.3.3. REASONS FOR MICROENCAPSULATION

There are several reasons for microencapsulation of active ingredients. In certain cases, the core has to be isolated from its surroundings; however in other cases the aim is to control the release from MCs (7). The main reasons for microencapsulation are (7, 13):

- Protection against UV, heat, oxidation, acids, bases and other active components
- Control of hygroscopy
- Enhanced dispersibility and flowability
- Enhanced solubility
- Handling liquids as solids
- Controlled (delayed or long-acting) and targeted release of active ingredients
- Enhanced penetration for a better efficacy
- Microencapsulation allows mixing of incompatible compounds
- Masking of taste and odours
- Microorganism and enzyme immobilization
- Enhanced visual aspect and marketing concept

Different intentions of MC demand different characteristics of MC. When selecting raw materials for microencapsulation and the suitable method, the most important parameters to be considered are: size and type of MC, chemical properties of shell material, degradability, biocompatibility and the permeation characteristics. The purpose of MCs is usually determined by its permeability. MCs with **impermeable shell** are preferred for those products where active ingredients have to be protected with no intend to influence the release kinetics. The effects reached by this type of MC include: protection of sensitive

ingredients toward environment, reduced instability of highly unstable ingredients, separation of reactive components, converting liquid ingredients into solid state, toxicity reduction and taste and odor masking. On the other hand, MCs with **permeable shell** are selected to modify release of active ingredients, especially for prolonged release of perfumes, deodorants, etc., or immobilization of ingredients with locally limited activity (7).

1.3.4. RELEASE MECHANISMS

Release mechanisms of encapsulated ingredients depend on the intention of microencapsulation and are planned in advance. The mechanism of external pressure is the first developed and still often used mechanism, which breaks the shell of MC and releases the liquid material from the core. The shell of MC may also breaks due to inner pressure. Shell can be dissolved in water or at selected pH value. Ingredients of core may be released also by abrasion of the MC shell. Additionally, core ingredients can be released due to increased temperature (cosmetic ingredients are released at body temperature), based on effect of melting the MC shell (7).

1.3.5. MANUFACTURING METHODS

Regardless of the MC formation approach, any of the methods can be divided into three basic steps (9):

1st step: Incorporation of the active substance in the system, which will later form the core of MC; system can be in form of a solution, emulsion or suspension (9).

2nd step: formation (production) of MC;

a) In case of liquid core material, the latter is dispersed in air (with the process of extrusion of liquid or spray process), in the second fluid (process of emulsification or microemulsification) or in the supercritical fluid.
b) In case of a solid core material, coating solution is sprayed on moving solid particles (fluid bed coating, coating in drum) (9).

3rd step: Stabilization/consolidation of produced MCs by chemical (polymerization), physical-chemical (gelation/cross-linking, coacervation) or physical methods (drying, precipitation, hardening), which leads to the formation of a solid cosmetic form (9).

There are many factors to consider when selecting the encapsulation process.

The method selected for encapsulation should always depend on the type and physical properties of the core and shell material and on the purpose of use; the latter is predominantly governed by core material of the encapsulated substances (8, 14). The chosen encapsulation method should (8, 12, 14):

- give high encapsulation efficiency, loading capacity of active ingredients and high production
- not exhibit aggregation or adherence of MCs
- give small (less than 0.7 mm), mono-dispersed, homogenous and spherically shaped capsules
- give a narrow size distribution without trails, threads or dents on the surface
- produce capsules in a short production time
- be suitable for industrial scale production
- allows different production size
- have the ability to produce MCs under sterile conditions if required.

Many different methods for production of MCs have been described. The methods can be categorized as chemical, physicochemical or mechanical process and include the following examples (8, 12) (Figure 4):

- **Chemical:** *in situ* polymerization and interfacial polymerization
- **Physiochemical:** coacervation
- **Mechanical:** spray-drying and extrusion-based methods

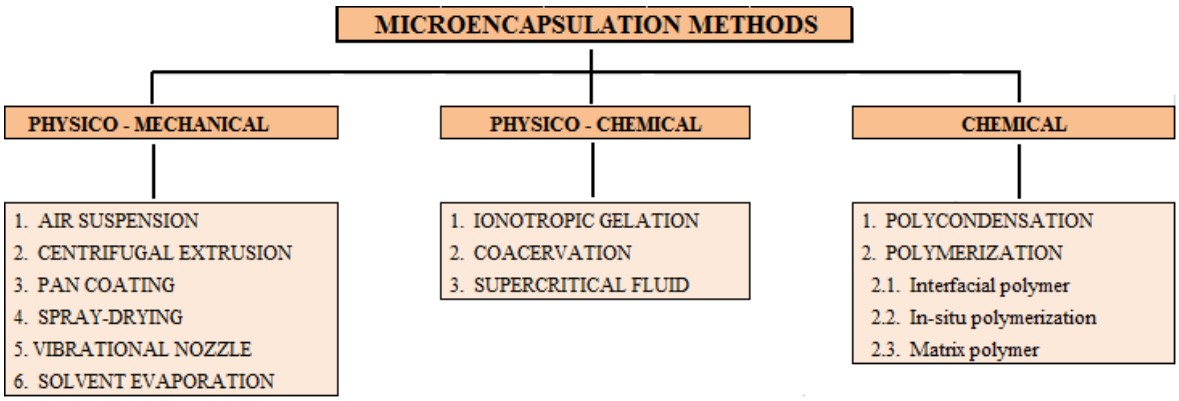


Figure 4: Different microencapsulation methods.

1.3.5.1. MECHANICAL METHODS

Mechanical methods are most frequently used for producing MCs. These methods are based on the principle of generating droplets from a polymer extruded through a nozzle and work using mechanical means (vibration forces) to increase the normal dripping process at the nozzle, or they break-up the extruded liquid stream produced by the polymer when it is passed through the nozzle. After production, the droplets are immediately solidified to capsules by either physical (cooling, heating) or chemical means (gelation). Extrusion of a polymer through a nozzle is used mainly at a lab scale. The main mechanical methods for fluid dispersion into droplets and subsequent conversion into capsules are: coaxial air-flow, electrostatic extrusion, rotating disc, jet-cutting, spray-draying, vibrating nozzle (8).

Technical background

At the nozzle outflow five different mechanisms of droplet formation as a function of the jet outflow velocity take place (Figure 5). These mechanisms arise over the interaction of gravity, friction forces impulse and surface tension (12).

Mechanism 1 presents direct formation of single droplets at the outflow orifice due to low outflow velocity. The droplet diameter d_d is calculated from surface tension and gravity (12) (Equation *1*):

$$(1) \qquad d_d = \sqrt[3]{\frac{6 d_n \sigma}{g \Delta \rho}}$$

Mechanism 2 presents uninterrupted outflow of the jet, because of increased kinetic force. Afterwards outflow of the jet breaks up due to axial symmetrical vibration and the surface tension. A further jet velocity increase leads to statistical distribution of the droplet size. Such distribution can be caused by spiral symmetrical vibration, presented in mechanisms 3 and 4, or by the high friction forces as presented in mechanism 5 (12).

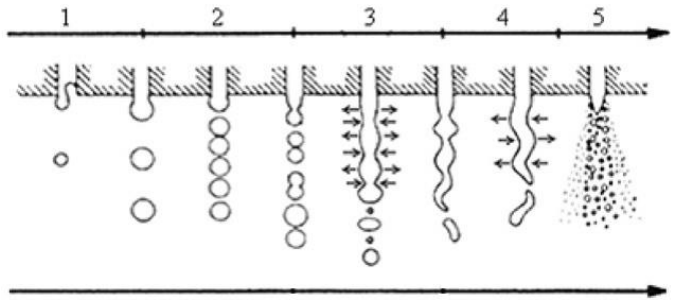


Figure 5: Different mechanisms of droplet formation as a function of jet velocities (4).

1.3.5.1.1. Vibration nozzle method

The vibration nozzle (VN) method, which is more commonly known as the vibrating-jet method or prilling, is one of the most widely used methods for the production of MC. The principle of VN method is based on breaking-up a laminar jet by the application of vibration frequency with defined amplitude to the extruded jet (8).

In the late 19th century, the volatility of liquid jets was theoretically analysed by Lord Rayleigh (12). He demonstrated that controlled break-up of laminar jet into uniform and equal size droplets can be achieved simply by applying a permanent sinusoidal force at defined frequencies to the jet. The result is the formation of droplet per hertz of frequency applied (Figure 6). The sinusoidal force is applied by vibrating the nozzle (8). He also demonstrated that the maximum volatility of frequency is correlated with the nozzle diameter and the jet velocity (12) (Equations: *2, 3, 4*).

$$\lambda = \frac{v_j}{f} \qquad (2)$$

$$\lambda_{opt} = \pi d_n \sqrt{1 + \frac{3\eta}{\sqrt{\rho \sigma d_n}}} \qquad (3)$$

$$d_d = \sqrt[3]{1.5 d_n^2 \lambda_{opt}} \qquad (4)$$

The optimal vibration parameters are defined in the light of the encapsulator stroboscope. Once the parameters are defined, they can be reset in the future, which makes the process highly reproducible. Diameter of the bead can be set between 0, 1 – 1, 5 mm (12). The selected vibration frequency determines the amount of the droplets produced, for example a vibration frequency of 7000 Hz generates 7000 droplets per second (15).

The properties of the formed drops depend on the nozzle diameter, the size of the frequency at defined amplitude, the viscosity of the extruded liquid and its surface tension and the flow rate of the laminar jet (8).

Although vibrational system is theoretically based on Newtonian liquids, it can also be applied to non-Newtonian liquids such as alginate, to make uniform droplets, which can be produced into MCs by methods such as ionotropic gelation (8). For most polymers, it must be noted, that the size of the droplet does not necessarily equal the size of the fabricated MCs (8).

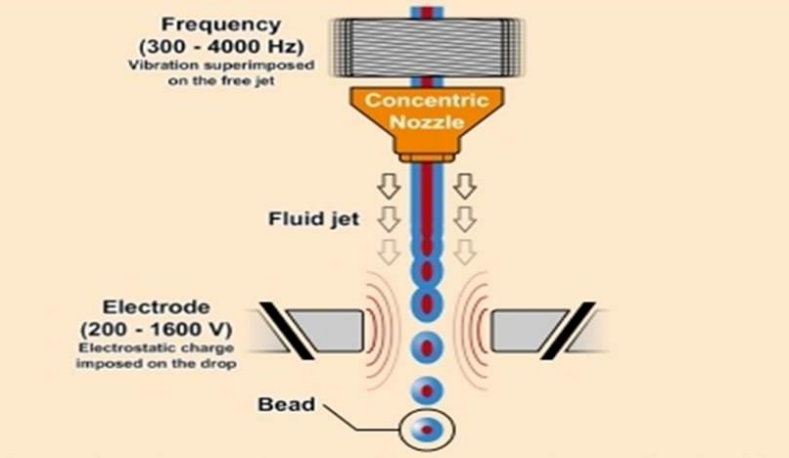


Figure 6: Formation of microcapsules by vibration nozzle method using concentric nozzle system (5).

Concentric nozzle system

The concentric nozzle system consists of the two single nozzles termed an internal and an external nozzle, in which the inner nozzle is placed directly into the outer one (Figure 7). The external nozzle can be anywhere between 50 and 900 μm wider compared to the internal nozzle, which enables the membrane size to be controlled (membrane size can also be determined by varying the volume of the shell/core material). Single core MCs can be described as having the average diameter (d_m), which is the sum of the diameter of the core and the shell material and an average internal diameter (d_c), which consists solely of the

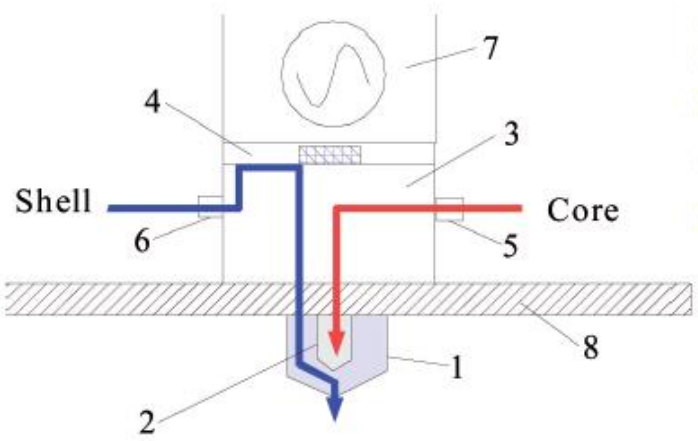
measurement of the core diameter. The average size (thickness) of the MC membrane (M_m) can then be obtained from the following equation: (8)

$$Mm = \frac{d_m - d_c}{2} \quad (5)$$

Challenges facing the VN system (8):

- Small production yields of MCs – increasing the production rates can be obtained by simply increasing the number of nozzles on the machine.
- Limited availability of polymers for producing the desired MCs.

It is envisaged that in the future, encapsulating devices applied at an industrial level could possibly contain several hundred nozzles, which would enable the required quantities of tons/day to be produced (8).



1	Shell nozzle
2	Core nozzle
3	CN pulsation body
4	Magnet holder
5	Luer connection for core liquid
6	Luer connection for shell liquid
7	Vibration unit
8	Carrier plate

Figure 7: Schematic description of the concentric nozzle system (6).

2. AIM OF THE WORK

The aim of the diploma thesis is to produce natural microcapsules by vibrational nozzle method as a delivery system for cosmetic active ingredients such as antioxidants, to increase solubility and achieve controlled release. Microcapsules are composed of shell and core. For shell material we will use sodium alginate and for core material we will use solution of lipophilic active ingredient in oil or hydrophilic active ingredient in W/O emulsion. We will try to find optimal processing parameters to produce mononuclear spherical microcapsules with narrow size distribution, optimal shell thickness and high degree of encapsulation and encapsulation efficiency.

Optimal process parameters will be indicated by visualization of bead formation in the encapsulator stroboscope. Morphology, shell thickness and degree of encapsulation will be determinated by light microscope, particle size distribution by laser diffraction and encapsulation efficiency by UV-Vis spectrophotometer.

3. EXPERIMENTAL PART

3.1. MATERIALS

3.1.1. COMPOSITION OF CARRIER SYSTEM: ALGINATE MICROCAPSULES

- **Alginate** – $(C_6H_7NaO_6)_n$ (Low viscosity grade – Büchi, Switzerland)

Alginate has been used successfully in the cosmetic industry as a gelling agent and colloidal stabilizer, and holds strong potential in the area of drug delivery (16). The main reasons that led to the choice of alginate are: biocompatibility and biodegradability, because can be broken down under normal physiological conditions; usually it does not require use of organic solvents for the preparation of MCs; the microencapsulation process takes place in mild conditions, suitable for the encapsulation of protein drugs or cells (15).

Sources of alginate

Alginate is extracted from various species of bacteria and kelp - brown algae including *Laminaria hyperborean*, *Ascophyllum nodosum*, and *Macrocysis pyrifera* (16, 17). The alginate forms mixed salts with various cations naturally found in sea water and the native species is usually found as an insoluble Ca^{2+} cross-linked gel (16).

Alginate chemistry

Alginate polymers consist of linear, nonbranched polysaccharides with acid residues of 1,4'-linked β-D-mannuronic acid and α-L-guluronic acid residues. The residues are arranged in blocks along the chain and vary in sequence and composition (16) (Figure 8).

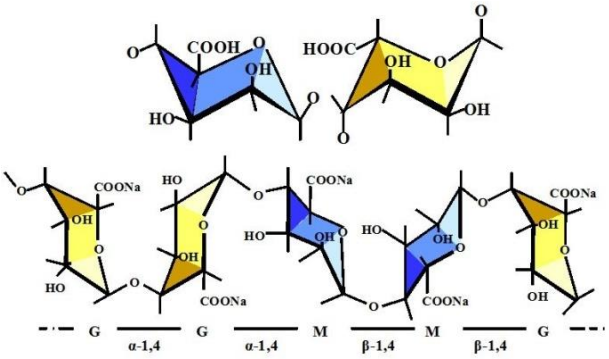


Figure 8: The chemical structure of alginate with β-D-mannuronic (M) acid blocks and α-L-guluronic (G) acid blocks (7).

It is known that gelification of alginate chains is due to strong interactions between calcium ions and guluronic acid residues. For this reason alginate polymers with high guluronic acid content (G blocks) form hard gels that are stable at high temperature, whereas polymers with high mannuronic acid content (M-blocks) form elastic gels more stable to freeze and thawing process (15). Increased alginate concentrations can result in MCs with considerably improved mechanical properties. However, exponential increases in viscosity are obtained with increasing concentrations of alginate. High viscosities can prevent extrusion of the alginate through the nozzle (8).

Sol-gel transformation

In general, alginate beads are formed when a solution of sodium alginate and the desired substance is extruded as droplets into a divalent solution to encourage cross-linking of the polymers. Generally, the formation of gel can occur by three mechanisms: external gelation, internal gelation and cooling (17). In the gelation process the polymer chains are cross-linked by the exchange of sodium ions from gluronic acids with divalent cations, forming what is referred to as the "egg-box" (16) (Figure 9). In the case of Ca^{2+} ions, the chelation at the G-residue of the alginate molecules results in ionic interaction between the guluronic acid groups, while the van der Waals forces between alginate segments result in three-dimensional gel network (16, 17).

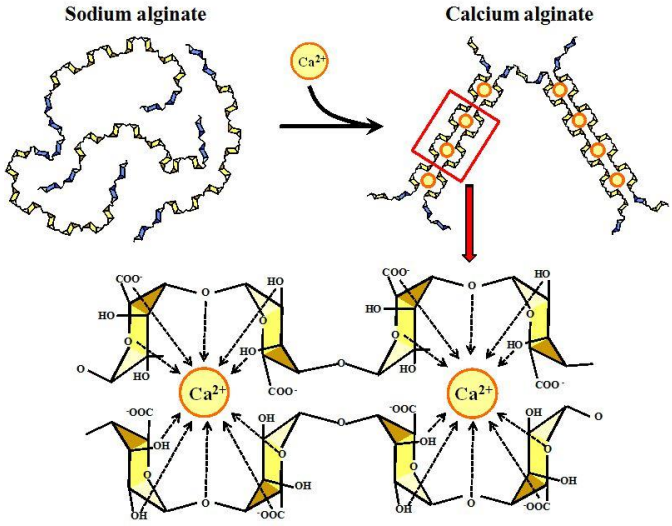


Figure 9: Formation of "Egg-box" (7).

The viscosity of alginate influences the diameter and the shape of the bead produced. As the concentration of the sodium alginate is increased, the produced beads become larger and more spherical (16).

Alginate matrices contain aqueous internal environments ideal for the encapsulation of many different substances. These encapsulations have high rate of substance diffusion due to their porous gel state that is controlled trough coating procedure. In addition, alginate matrices are very biodegradable and can be broken down under normal physiological conditions (16).

- **Resveratrol** – $C_{14}H_{12}O_3$ (Sigma Aldrich, Germany)

Resveratrol (RSV) (3, 5, 4'-trihydroxystilbene or 3, 5, 4'-stilbenetriol; MW: 228, 25) is a non-flavonoid polyphenolic compound (Figure 10), produced by several plant species, such as grapes, blueberries, cranberries and peanuts. It was first detected in the roots of white hellebore (*Veratrum grandiflorum*) in 1940, and later in 1963, isolated from the roots of *Polygonum cupsidatum*. RSV is usually extracted from red grapes skin, which contains about 50-100 μg of RSV per gram. RSV is a fat-soluble compound that naturally exists in the *cis*- and *trans*- stereoisomeric forms. The trans- form is the preferred steric form in nature and is relatively stable. It can undergo isomerisation to the cis-form when exposed to ultraviolet (UV) irradiation (18, 19, 20).

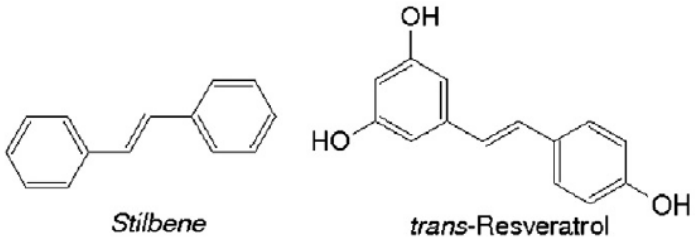


Figure 10: Chemical structure of stilbene and trans-resveratrol (8).

RSV has antioxidant, anti-inflammatory and chemopreventive actions (19). Studies have shown protective effects against UV-radiation, which protect skin from oxidative stress and cutaneous damages including skin cancer (21). RSV has been shown to have low toxicity and limited side effects (21).

It was shown that topical application of RSV (10μmol/0.2 ml) to SKH-1 hairless mice results in significant inhibitions of UVB irradiation resulted in significant decrease in

skin edema and hyperplasia, inflammation, cyclooxygenase (COX) and ornithine decarboxylase (ODC) induction, generation of hydrogen peroxide (H_2O_2) and lipid peroxidation, and infiltration of leukocytes and protein levels of proliferating cell nuclear antigen (PCNA) in the skin (21).

- **Beta-carotene** – $C_{40}H_{56}$ (Fluka Sigma Aldrich, Germany)

Beta-carotene is chemically classified as a hydrocarbon and specifically as a terpenoid (isoprenoid), reflecting its derivation from isoprene units (Figure 11). It is a yellow pigment found in chlorophyll-containing plants, bacteria, and food such as carrots, tomatoes, spinach, etc. Among 600 known carotenoids, the substance is unique, because two molecules of retinol (vitamin A) can form beta-carotene (22).

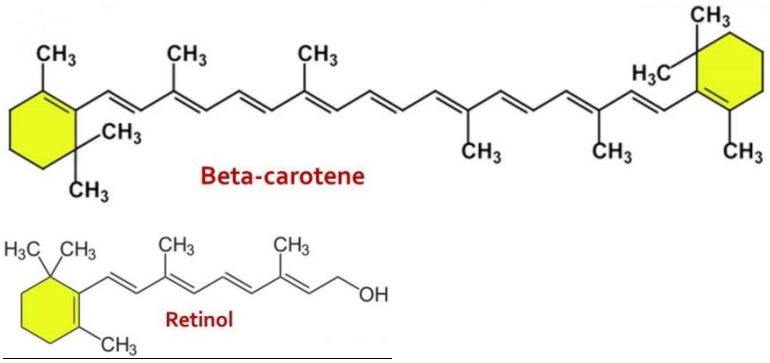


Figure 11: Chemical structures of beta-carotene and retinol (9).

Beta-carotene is much more bioavailable in its crystalline form than in its naturally occurring form (22).

As a precursor of vitamin A, beta-carotene is a potent lipid–soluble antioxidant capable of quenching radicals, especially singlet oxygen, but also superoxide anion and hydroxyl radicals. Singlet oxygen is capable of inducing DNA damage and is thus mutagenic. Beta-carotene has been shown to have topical photoprotective effects at 0.05 % concentration. It protects skin against UVA radiation effects by decreasing lipid peroxyl radicals. Therefore, it is used to treat solar urticaria, polymorphic light eruptions, hydroa vacciniforme, lupus erythematosus and photoallergic drug reactions. The primary benefit of beta-carotene in cosmetic products has been its ability to normalize keratinization. Recent evidence suggests that beta-carotene may maintain collagen integrity and immune defense by enhancing cytotoxicity of macrophages against tumor cells. Therefore, it might

have an impact on carcinogenesis (1, 22). It is also used in cosmetics as a sunscreen to prevent acute sunburn reactions and erythema (premature aging of skin) (22, 23).

- **Calcium Chloride dihydrate** – $CaCl_2 * 2H_2O$ (Sigma Aldrich, Germany)

Calcium chloride occurs as a white or colourless crystalline powder, granules, or crystalline mass, and is hygroscopic (deliquescent). The dissolution of calcium chloride in water is an exothermic reaction. Calcium chloride has been used to control the release of active ingredients from solid topical or oral dosage forms by crosslinking sodium alginate or by its interaction with chitosan. It is GRAS (generally recognized as safe) listed and used in topical, ophthalmic, and injection preparations (injections, suspensions, creams, eye drops, intraocular irrigation, vaccines, nebulizer solution, oral suspension, etc.) (24).

- **Almond oil –** C_7H_6O (Fagron, Netherlands)

Almond oil is pressed from the seeds of the bitter or sweet almond, *Prunus amygdalu,* var. *amara* or var. *dulcis*. It consists mostly of glycerides of oleic acid, with smaller amounts of linoleic and palmitic acid. It is clear, colourless or pale-yellow colored oil with a bland, nutty taste. Almond oil is used as an emollient, oleaginous vehicle and solvent (24).

- **Chitosan** – $(C_8H_{13}NO_5)_n$ (Sigma Aldrich, Germany, high purity, M_v 60,000-120,000)

Chemical name of chitosan is poly-β-(1,4)-2-amino-2-deoxy-$_D$-glucosamine. Chitosan is manufactured by chemically treating the shells of crustaceans, such as shrimps and crabs. Partial deacetylation of chitin results in the production of chitosan, which is a polysaccharide comprising copolymers of glucosamine and *N*-acetlyglucosamine. It is available in several types and grades that vary in molecular weight by 10 000 – 1 000 000, and vary in degree of deacetylation and viscosity. Chitosan occurs as odourless, white or creamy-white powder or flakes. It is used in cosmetics and in pharmaceutical formulations as coating agent, disintegrant, film-forming agent, mucoadhesive tablet binder, viscosity increasing agent. Chitosan has been processed into several forms including gels, films, beads, microspheres, tablets and coating for liposomes. Furthermore, chitosan may be processed into drug delivery systems (24).

- **Acetic acid** – CH_3COOH (Cooper, India; 85-90% (w/w) solution in water)

Chemical name of acetic acid is ethanolic acid. Acetic acid solution is widely used as acidifying agent in a variety of pharmaceutical formulations. It is also claimed to have some antibacterial and antifungal properties (24).

- **Tween 20** – $C_{58}H_{114}O_{26}$ (Fagron, Netherlands)

Tween 20 or polysorbate 20 is a polyoxyethylene derivative of sorbitan monolaurate. It occurs as clear, yellow to yellow-green viscous liquid. Polysorbate 20 is used as an excipient in pharmaceutical applications to stabilize emulsions and suspensions. Its hydrophile-lipophile balance (HLB) is 16.7; this indicates that the surfactant will travel into the water phase (25).

- **Distilled water**

Destilated water was obtained by the process of distillation at the Faculty of pharmacy, University of Mediterranean (Aix-Marseille II).

- **Labrafac CC** (Gattefossé, France)

Labrafac CC is caprylic/capric triglyceride of crystal-clear grade. Labrafac CC is a universal emollient and solubilizer for oils and emulsions and vehicle for capsule formation. It improves the feeling and spreadability of cosmetic products on the skin (26, 27).

- **Polyglycerol-3-diisostearate** (Gattefossé, France)

Polyglycerol-3-isostearate is a liquid, non-ionic W/O surfactant based on linear polyglycerol and fatty acids. It meets the requirements of modern cosmetic ingredients, such as: PED-free, can be used cold, non-nitrosamine forming, toxicologically safe, based on natural raw materials and good biodegradability, because can be broken down under normal physiological conditions (28).

- **Glycerol** – $C_3H_8O_3$ (Fagron, Netherlands)

In topical pharmaceutical formulations and cosmetics, glycerine is used primarily for its humectant and emollient properties. Glycerine is used as a solvent or cosolvent in creams

and emulsions. It is a clear, colorless, odourless, viscous, hygroscopic liquid. Glycerine is mainly obtained from oil and fats as a by-product in the manufacture of soaps and fatty acids (24).

- **Propylene glycol** - $C_3H_8O_2$ (Fagron, Netherlands)

Propylene glycol has been widely used as a solvent, preservative, plasticizer and especially in cosmetics as carrier for emulsifiers. It is a clear, colorless, odourless, viscous liquid (24).

- **Beewax** - $C_{15}H_{31}COOC_{30}H_{61}$ (Fagron, Netherlands)

Beewax is a natural wax produced in the bee hive of honey bees of the genus *Apis*. Its main components are esters of fatty acids and various long-chain alcohols. In cosmetics beewax is widely used as a cream barrier and moisturizer (29).

3.2. METHODS

We used the Büchi Encapsulator B-390 with concentric nozzle system for microencapsulation of liquid formulations with antioxidants (Figure 12). It is a semi-automated instrument used for polymer encapsulation of chemical substance, bio-molecules, drugs, flavour and fragrances, pigments, extracts, cells and microorganisms under open conditions. Aseptic working conditions are possible, because all parts in contact with the encapsulation mixture are autoclavable.

The bead formation is based on the fact that a controlled, laminar liquid jet is broken into equally sized beads, if vibrated at an optimal frequency. The Encapsulator B-390 provides just such controlled conditions to generate beads between 0.15 to 2 mm (30).

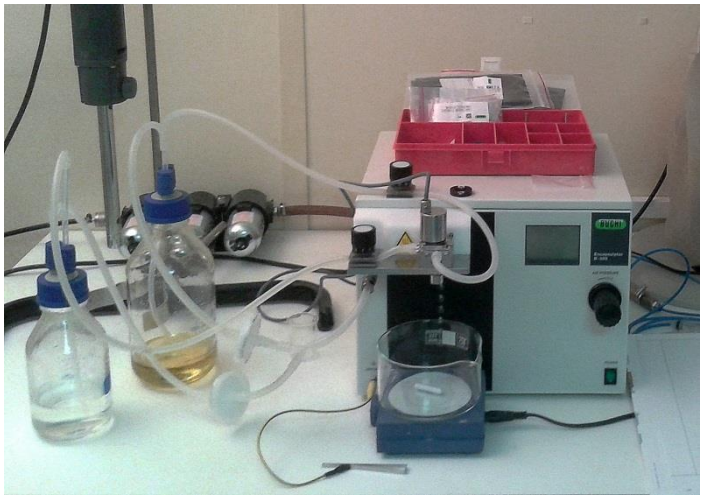


Figure 12: Büchi Encapsulator B-390

3.2.1. PREPARATION OF SAMPLES

- **Shell solution**

We prepared 2 % (w/w) solution of low viscosity grade sodium alginate in distillated water. Water was mixed by VMI Rayneri mixer and sodium alginate powder was slowly added. We mixed till we got homogenous solution. Sodium alginate solution was placed in the fridge overnight at 4°C to remove air bubbles. The next day solution was degased by Fisher Scientific FB 15061 ultrasonic bath. We measured viscosity of sodium alginate solution by Brookfield viscometer DV-II + PRO.

- **Core solution**

Solution of Rouge covapate W 3773

We prepared 0.1 % (w/w) solution of Rouge covapate W 3773 in almond oil to get coluored core phase. With this solution, determination of appropriate concentration of hardening solution and process parameters for efficient microencapsulating process was easier.

Solution of beta-carotene

We prepared 0.1% (w/V) solution of beta-carotene in almond oil. Solution was mixed by magnetic stirring.

Solution of resveratrol

Resveratrol did not completely dissolve in almond oil, therefore we added Labrafac CC, which improves its solubility. We prepared 0.1 % (w/V) solution of RSV in almond oil (50% (w/V)) and Labrafac CC (50% (w/V)). Solution was mixed by magnetic stirring and afterwards sonificated by Fisher Scientific FB 15061 ultrasonic bath for 30 min.

Water in oil (W/O) emulsion with hydrophilic antioxidant niacinamide

To get stable W/O emulsion, we prepared many emulsions with different ingredients and different concentrations of lipophilic phase. For encapsulation of emulsion by vibration nozzle method, which uses alginate as shell material, emulsion have to has at least 80 % of lipophilic phase, due to possible interactions between alginate and water from emulsion. However, we prepared W/O emulsion with 45% of lipophilic phase and encapsulation was still successful (Table III).

Ingredients of lipophilic phase were mixed together in a beaker by magnetic stirring at melting point of beeswax, 65°C. Ingredients of hydrophilic phase were mixed together in a beaker by magnetic stirring at 65°C as well. Afterwards, we slowly added hydrophilic phase to lipophilic phase; mixing by magnetic stirring proceeded all the time of adding hydrophilic phase. Emulsion was then homogenised with IKA T25 digital Ultra-Turrax homogenizer, till emulsion cooled down to room temperature.

For determination of emulsion type we measured conductivity of emulsion by conductivity meter, Model 627 by Scientifica. The measurement ranges from 20 to 20 000

μSiemens/cm. Low conductivity suggests W/O emulsion and high conductivity indicates O/W emulsion. Measured conductivity for the emulsion was 0.3 μS/cm, which corresponds to W/O type.

Table II: Formulation of W/O emulsion.

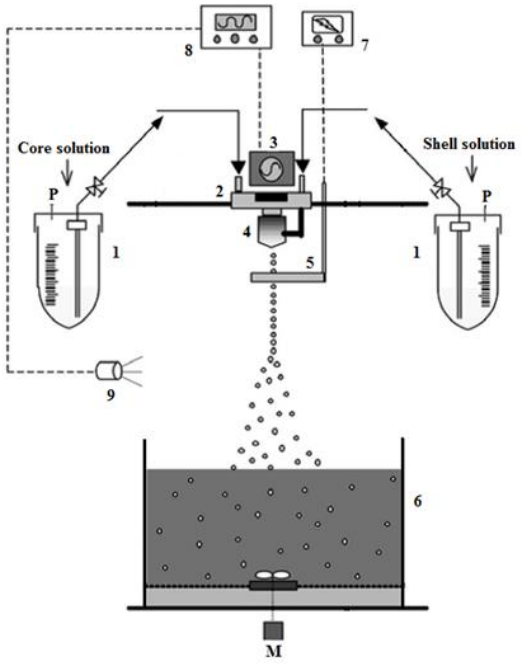
A - Lipophilic phase	B – Hydrophilic phase
Almond oil ~ 40 %	Glycerin ~ 3.5 %
Polyglycerol-3-diisostearate ~ 4 %	Propylene glycol ~ 1.5 %
Beewax ~ 1 %	Water ~ to 100 %

- **Hardening solution**

Different concentrations of calcium chloride solutions were prepared: 0.5 M; 1 M; 1.5 M; 2M; 2.5 M. When we determined which solution of $CaCl_2$ is the most appropriate, 0.1 % (w/V) solution of chitosan in acetic acid was added. Chitosan has ability to retain core phase inside MC during drying process (9). Additionally, Tween 20 was added to eliminate surface tension, which can cause oval shape of MCs when entering into hardening solution (30). Additionally, we tried to determinate the most optimal time of how long to let MCs in hardening solution, wherein hardening solution is responsible for hardness of MC shell.

3.2.2. MICROENCAPSULATION PROCESS

The main parts of the encapsulator with concentric nozzle system are shown in Figure 13.



1	product delivery bottle
2	pulsation chamber
3	vibration system
4	concentric nozzle
5	dispersion system
6	reaction vessel
7	electrostatic charge generator
8	frequency generator
9	stroboscope
M	magnetic stirrer
P	pressure control system

Figure 13: Schematic representation of the concentric nozzle lab scale encapsulating system (10).

The core and the shell materials were put into product delivery bottles. Both liquids were forced to the concentric nozzle by air pressure (P). The liquids passed through a precisely drilled concentric nozzle, resulting in formation of a co-extruded laminar liquid jet, which was broken up into mononuclear drops by the application of a vibrational frequency. These droplets passed an electrical field between the concentric nozzle and the electrode, resulting in a surface charge. Generated MCs were collected in a hardening solution within the reaction vessel. Solution in a reaction vessel was continuously mixed by a magnetic stir bar (M) to prevent clumping (8, 15). After some time the hardening solution was drained off and the MCs were washed by distilled water.

For core material we first used 0.1 % (w/w) solution of Rouge covapate W 3773 in almond oil to get coloured core phase. With this solution, determination of appropriate concentration of hardening solution and process parameters for efficient microencapsulation process was easier.

3.2.3. DETERMINATION OF OPTIMAL PROCESS PARAMETERS

To set up an effective microencapsulation process, leading to well-formulated MCs, we had to determinate appropriate parameters.

Most of parameters for optimal MC formation were indicated by visualization of real-time bead formation in the light of stroboscope lamp. When optimal parameters were reached, a steady chain of droplets was clearly visible. Once established, the optimal parameters can be present for subsequent bead production runs with the same encapsulating mixture (30).

PHYSICAL PARAMETERS

Viscosity of liquids: 0 - 10 000 mPa*s

Concentration of hardening solution: 0.5 – 2.5 M

PROCESS PARAMETERS

Inner (core) nozzle: 0.08, 0.12, 0.15, 0.20, 0.30, 0.45, 0.75 and 1.00 mm.

Outer (shell) nozzle: 0.20, 0.30, 0.40, 0.50, 0.60, 0.70 and 0.90 mm.

Electrode: is a part of electrostatic dispersion unit. When the beads pass through the electrode they pick up the electrostatic charge. Increased voltage causes circular dispersal of the bead stream after the electrode. The repulsion forces induced by the equally charged surfaces of droplets prevent coalescence. It can be set from 250 to 2500 V. The applied voltage depends primarily on the bead size and the liquid flow velocity. The larger are beads, the higher electrostatic voltage is needed to separate the jet. The distance between the electrode and the nozzle tip has to be approximately 3 to 8 mm (30).

Vibration frequency: generates the appropriate electric oscillation in the vibration unit. It can be set from 40 to 6,000 Hz. Higher frequencies generate smaller bead sizes. The selectable vibration frequency determines the quantity of the droplets produced; for example, 1.000 droplets are generated per second at 1.000 Hz (30).

Amplitude: is the intensity of vibration. By increasing the amplitude vibration becomes stronger. It can be set from 1 to 12 (30).

Flow rate: can be set from 0.05 to 200 mL/min. Lower liquid flow rates generate smaller bead sizes. To obtain good production conditions the flow rate of the shell material is usually at least twice that of the core liquid.

Pressure: maximum is 1.5 bar.

Heating: can be set from 30°C to 70°C. The higher is the process temperature, the smaller the bead size.

Stirring speed in hardening bath: The higher the stirring speed, the smaller the bead size.

Distance between nozzle and hardening solution: 2 - 10 cm.

Hardening time of microcapsules in hardening solution

3.2.4. DETERMINATION OF MICROCAPSULES PROPERTIES

3.2.4.1. SIZE DISTRIBUTION

Size was determined by high-resolution laser granulometer (Mastersizer 2000, Malvern Instruments Ltd, England). Instrument uses the technique of laser diffraction, which is based on the principle that particles passing through beam will scatter light at an angle that is directly related to their size (Figure 14). Large particles therefore scatter light at narrow angles with high intensity, whereas small particles scatter at wider angles but with low intensity. It does this by measuring the intensity of light scattered as a laser beam. These data are than analysed to calculate the size of the particles that created the scattering pattern (31).

MCs samples were suspended in water at room temperature and were analysed.

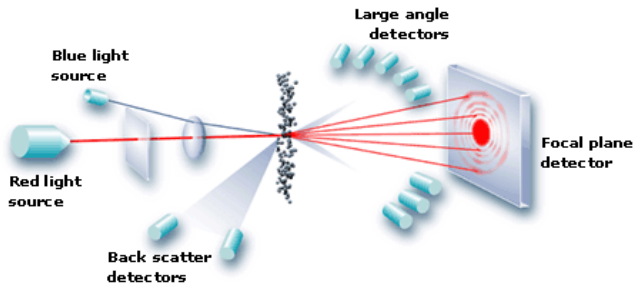


Figure 14: Laser diffraction as a measure principle of Mastersizer (11).

Results of particle size analysis are reported as $d_{0.1}$, $d_{0.5}$ and $d_{0.9}$ µm values corresponding to the particle percentages below the stated number, and span values that define the particle size distribution according to the following equation:

$$(6) \qquad Span = \frac{d_{0.9} - d_{0.1}}{d_{0.5}}$$

The size span corresponds to the ratio of the largest and smallest sizes. The span measures the width of the principle size distribution. A small span value indicates a narrow particle size distribution.

3.2.4.2. MORPHOLOGY, SHELL THICKNESS AND ENCAPSULATION DEGREE [%] OF MICROCAPSULES

These properties were investigated by a light microscope (Lecia CM E, Milano, Italy) at 200x magnification on freshly prepared MCs. All images of MCs were taken using a camera attached to a light microscope, interfaced to a PC.

For determination of encapsulation degree and shell thickness, diameters of MCs and MCs cores were measured by micrometer of light microscope.

Shell thickness

Single core MCs can be described as having an average diameter (d_m), which is the sum of the diameter of the core and shell material and an average internal diameter (d_c), which consists solely of the measurement of the core diameter (Figure 15). The average size (thickness) of the MC membrane (M_m) was obtained from the following equation (8):

$$(7) \qquad Mm = \frac{d_m - d_c}{2}$$

The thickness of the membrane material can be predetermined and controlled within a certain range for a given system by varying the ratio of the flow rate of the shell material to the flow rate of the core material in the extruded concentric liquid jet during the production process (8).

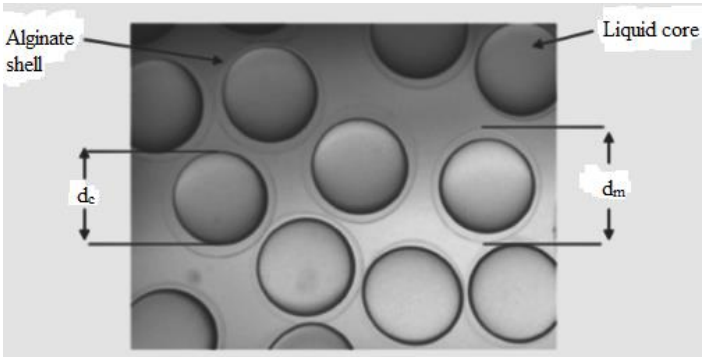


Figure 15: Light microscope image of microcapsules represents microcapsule and liquid-core diameters (4).

Encapsulation degree [%]

Degree of encapsulation determines what percentage of MC volume takes volume of core (Equation *8*). The highest is the degree of encapsulation, the thinner the shell, which allows efficient release of active ingredient from MC.

Volumes of MCs and cores were calculated from radius of MCs and cores by equitation of sphere volume.

$$(8) \qquad Encapsulation\ degree\ [\%] = \frac{V_c}{V_{MC}} * 100\%$$

3.2.4.3. ENCAPSULATION EFFICIENCY

Encapsulation efficiency was investigated by UV-Vis spectrophotometer (Shimadzu Corporation, UV – 2401PC, Japan). Measurement was performed only for beta-carotene loaded MCs, because the same microencapsulation method was used for all active ingredients.

UV-Vis spectroscopy

Ultraviolet-visible spectroscopy refers to absorption or reflectance spectroscopy in the ultraviolet-visible spectral region. The absorption or reflectance in the visible range directly affects the perceived colour of the chemicals involved. Molecules containing π-electrons or non-bonding (n-) electrons can absorb the energy in the form of UV or visible light to excite these electrons to higher anti-bonding molecular orbitals. Compounds with easily excited electrons, absorb light of longer wavelength and vice versa (32).

A spectrophotometer is employed to measure the amount of light that a sample absorbs. A beam of light form a visible and/or UV light source is separated into its component wavelengths by a prism or diffraction grating. Each monochromatic beam is in turn split into two equal intensity beams by a half-mirrored device. The sample beam passes through a small transparent container (cuvette) containing solution of the compound being studied in a transparent solvent. The reference beam passes through an identical cuvette containing only the solvent. The intensities of these light beams are then measured by electronic detectors and compared (Figure: 16). The beam of light consists of photon stream and when they encounter an analyte molecule, there is a chance the analyte will absorb the photon. This absorption reduced the number of photons in the beam of light, thereby reducing the intensity of the light beam. The UV region scanned is normally from 200 to 400 nm, and the visible portion is from 400 to 800 nm (33, 34).

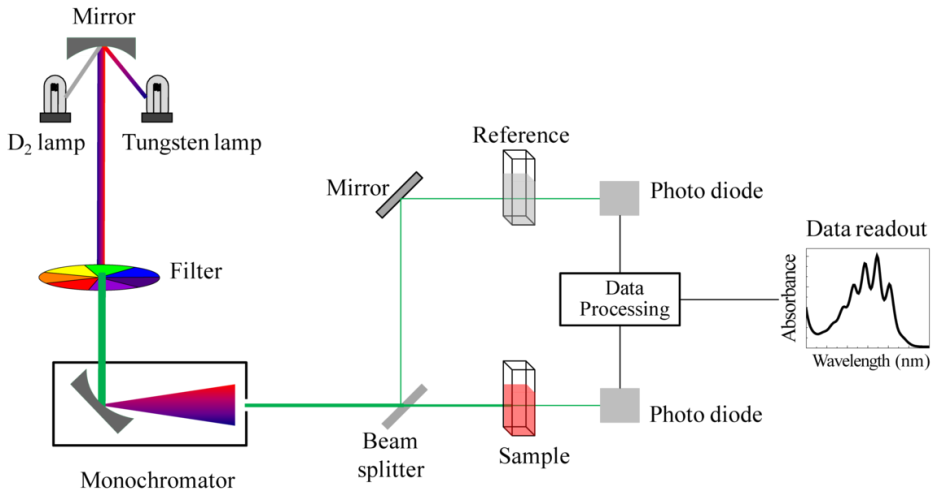


Figure 16: Schematic of UV-Vis spectrophotometer (12).

Measuring conditions for UV-Vis spectrophotometer

As a solvent we chose almond oil. Analysis was performed in a dark laboratory to avoid impact of light. All samples were measured at absorption maximum of beta carotene in oil solution, i.e. at wavelength λ_{max} = 461 nm. Measured concentrations were expressed in mg/L.

Preparation of calibration line

First, the intensity of light passing through a blank was measured. For preparation of calibration line we prepared 0.1 % (w/V) solution of beta-carotene in almond oil. The solution was afterwards diluted for dilution factor 10, to concentration 100 mg/L. This solution was afterwards diluted for dilution factors of 2, up to 6.25 mg/L. Solutions at different concentrations were transferred into cuvettes and absorbance was measured by spectrophotometer at 461 nm.

Preparation of samples of beta-carotene loaded microcapsules

For extraction of representative portion of beta-carotene from MCs, we mechanically crushed fresh MCs. Afterwards they were placed in a centrifuge tube and centrifuged at 2500 rpm for 30 minutes at 25°C by Eppendorf Centrifuge 5702 RH, France. Supernatant was collected and the remaining crushed MCs were centrifuged at 4400 rpm for 5 minutes at 25°C. Both supernatants were pooled in tube and extract was diluted with almond oil for dilution factor 10. Diluted extract was pipetted in cuvette and the absorbance was determined at 461 nm wavelength by UV-Vis spectrophotometer. UV-Vis absorbance cutoff wavelength of almond oil is 930 nm. If we compare absorbance wavelength of almond oil with this of beta-carotene, we find out that almond oil is appropriate solvent. Microencapsulation efficiency was determined by equation *9*. According to calibration line, we determined concentration of beta-carotene from microcapsules.

$$\text{(9)} \qquad \%\ \text{Microencapsulation efficiency} = \frac{C1}{C0} * 100$$

C_0 - concentration of beta-carotene in almond oil before microencapsulation

C_1 - concentration of beta-carotene in almond oil after microencapsulation

4. RESULTS AND DISCUSSION

In the diploma thesis, we wanted to identify parameters for production of liquid core microcapsules by the vibration nozzle method with a concentric nozzle. Different systems of core materials and different concentrations of shell and hardening solution were tested. Under reproducible production conditions, microcapsules showed narrow size distribution. By light microscope we determined microcapsules morphology, thickness of shell and degree of encapsulation. Encapsulation efficiency, which was determined by UV-Vis spectrophotometer, is one of the most important properties of microcapsules.

4.1. OPTIMAL PROCESS PARAMETERS FOR EFFICIENT MICROENCAPSULATION PROCESS

4.1.1. PHYSICAL PARAMETERS

Viscosity of sodium alginate solution

The limit of this microencapsulation method is the concentration of the polymer solution. We determined that viscosity of prepared 2 % (w/V) sodium alginate solution was 130.5 mPa*s, which corresponds to required conditions (i.e., maximum viscosity of used liquids should not exceed 10 000 mPa*s).

Higher polymer concentration corresponds to higher viscosity values and requires higher liquid jet flow rates that are not suitable to obtain regular and well-formed MCs (15). An increase in alginate concentration and consequently increased viscosity, allowed more Ca^{2+} biding sites and a greater number of alginate strands held together in the “egg-box” structure within the bead shell, thus resulting in a higher degree of cross-linking and a more rigid and compact matrix. Increased viscosity of alginate solution subsequently causes a slower diffusion of core substances (17). Therefore we had to find the most optimal viscosity of alginate solution, by which we could produce regular and well-formed MCs. Alginate solution, has to have enough yet not too much Ca^{2+} bidding sites for optimal cross-linking; this means that shell can control diffusion of actives ingredients from MCs. Too rigid and compact shell prevents diffusion of actives ingredients.

Concentration of hardening solution

We tested five different concentrations of hardening solution and after observing MCs, produced with different concentration of hardening solution under light microscope, we found, that the use of 1 M hardening solution produces the most optimal MCs.

Hardening solution is responsible for hardness of MCs shell. Low concentration of hardening solution produces soft and sensitive shell, which could cause faster and easier release of active ingredient from MCs. High concentration of hardening solution produces hard shell, which could prevent release of active ingredient from MCs. Larger amount of calcium salt produces greater crosslinkage and consequently thinner films (16).

4.1.2. PRODUCTION PARAMETERS

Size of nozzles

We tested different combinations of inner (IN) and outer nozzles (ON) (IN: 150µm + ON: 400 µm; IN: 200µm + ON: 600 µm; IN: 200µm + ON: 300 µm). Finally, we obtained the best process parameters by using the combination of 200 µm inner and 300 µm outer nozzles. The larger the size difference between nozzles, the higher flow rate is needed for core solution, which causes good rate of encapsulation. The smaller the size difference between nozzles, the more careful one has to be to obtain appropriate flow rate of core solution. If the flow rate is too high, microencapsulation is not possible, because the extruded volume of core solution is higher than the volume of shell solution adjustable on the encapsulator.

It must be noted that the size of the droplet does not necessarily equal the size of the fabricated MCs (8). The final MC diameter is approximately two times the nozzle size. In this experiment, MCs were produced by 300 µm outer nozzle, however the final average diameter of RSV and beta-carotene loaded MCs was 529.93 µm and 473.81 µm, respectively, whereas average diameter of W/O emulsion MCs was 489.24 µm. Although the same size of outer nozzle was used, average diameters are different, which corresponds to jet velocity and vibration frequency modification in each case. With changing these two parameters the range can be adjusted by about 1 – 15 % (30).

The final diameter of MC (d_m) can be calculated with the flow rate *V' [mL/min]* and the frequency of pulsation according to equation *10*:

$$(10) \qquad dm = \sqrt[3]{\frac{6\,V'}{\pi\,f}}$$

Determination of optimal concentration of hardening solution, vibration frequency, electrode tension, pressure/flow rate, amplitude and distance between nozzle and hardening solution

First experiments have been performed with Rouge covapate W 3773 in almond oil, only to learn how to operate with encapsulator and how to process parameters influence each other (Tables III, IV, V). In the first experiment we got very few MCs, because the concentration of hardening solution was too low, leading to weak cross-linking between sodium alginate and Ca^{2+} ions of hardening solution (Table III). We got well-formed MCs by using 1 M hardening solution and optimal process parameters (150 μm inner and 400 μm outer nozzle, vibration frequency at 420 Hz, electrode tension at 1300 V, pressure at 245 mBar and flow rate of shell solution 11.30 mL/min and core solution 1.20 mL/min; Table IV). Using 200 μm inner nozzle and 600 μm outer nozzle, vibration frequency at 420 Hz, electrode tension at 1800 V, pressure at 244 mBar and flow rate of shell solution 18.40 mL/min and core solution 1.50 mL/min, also led to well-formed MCs (Table V).

After initial experiments we learned how parameters affect MCs quality and have later tried to encapsulate actives ingredients, such as RSV and beta-carotene in almond oil. Two combinations of nozzles were used (150 μm + 400 μm, 200 μm + 300 μm) and in both cases well-formed MCs were produced (Table VI, VII). In the case of using 200 μm inner and 300 μm outer nozzles, the formed MCs were even better (the rate of encapsulation was higher, due to lower ratio between nozzles diameters). Vibration frequency at 800 Hz, electrode tension at 2500 V and pressure at 680 mBar, led to well-formed MCs (Table VII).

Encapsulating of W/O emulsion was completely different; the viscosity of emulsion was higher, hence process conditions as vibration frequency and the corresponding electrode tension needed to be reset. According to literature data, we heated alginate solution at 50 °C to reduce the viscosity and added Tween 20 (Table VIII, IX). Produced

MCs were satisfactory; however, if we used normal alginate solution as in all other experiments, MCs were even better. Finally, we produced MCs with two different nozzle combinations, as in the case of encapsulating active ingredient in almond oil (Tables X, XI). Very well-formed MCs were produced using 200 µm inner nozzle, 300 µm outer nozzle, vibration frequency at 420 Hz, electrode tension at 2500 V and pressure at 390 mBar (Table XI).

In all experiments amplitude was set to 6−7. Flow rate of shell solution was always at least twice that of core solution. The distance between nozzle and hardening solution was 8 cm.

Table III: Parameters used to encapsulate rouge covapate W 3773 in almond oil (inner nozzle: 150 µm, outer nozzle: 400µm; 2 % (w/V) sodium alginate; 0.5 M $CaCl_2 * 2H_2O$)

Frequency [Hz]	Electrode [V]	Pressure [mBar]	Results
420	2500	543	Few beads
420	1300	279	More beads, but still not desirable

Table IV: Parameters used to encapsulate rouge covapate W 3773 in almond oil (inner nozzle: 150 µm, outer nozzle: 400µm; 2 % (w/V) sodium alginate; 1 M $CaCl_2 * 2H_2O$)

Frequency [Hz]	Electrode [V]	Pressure [mBar]	Results	Flow rate [mL/min] of shell solution	Flow rate [mL/min] of core solution
420	**1300**	**245**	**Good**	11.30	1.20
240	2500	739	No beads		

Table V: Parameters used to encapsulate rouge covapate W 3773 in almond oil (inner nozzle: 200 µm, outer nozzle: 600µm; 2 % (w/V) sodium alginate; 1 M $CaCl_2 * 2H_2O$)

Frequency [Hz]	Electrode [V]	Pressure [mBar]	Results	Flow rate [mL/min] of shell solution	Flow rate [mL/min] of core solution
320	**1800**	**244**	**Good**	18.40	1.50

Table VI: Parameters used to encapsulate 0.1 (w/V) % solution of resveratrol or beta-carotene in almond oil (inner nozzle: 150 µm, outer nozzle: 400 µm; 2 % (w/V) sodium alginate; 1 M CaCl2 * 2H2O)

Frequency [Hz]	Electrode [V]	Pressure [mBar]	Results	Flow rate [mL/min] of shell solution	Flow rate [mL/min] of core solution
420	**2500**	**543**	**Good**	13.00	4.00

Table VII: Parameters used to encapsulate 0.1 (w/V) % solution of resveratrol or beta-carotene in almond oil (inner nozzle: 200 µm, outer nozzle: 300 µm; 2 % (w/V) sodium alginate; 1 M CaCl2 * 2H2O)

Frequency [Hz]	Electrode [V]	Pressure [mBar]	Results
420	2500	700	Good
800	**2500**	**680**	**Very good**

Table VIII: Parameters used to encapsulate W/O emulsion (inner nozzle: 150 µm, outer nozzle: 400µm; 2 % (w/V) sodium alginate - heating at 50°C + 0.2 (V/V) % Tween 20; 1 M $CaCl_2$ * $2H_2O$)

Frequency [Hz]	Electrode [V]	Pressure [mBar]	Results
420	1500	210	Good

Table IX: Parameters used to encapsulate W/O emulsion (inner nozzle: 150 µm, outer nozzle: 400µm; 2 % (w/V) sodium alginate + 0.2 (V/V) % Tween 20; 1 M $CaCl_2$ * $2H_2O$)

Frequency [Hz]	Electrode [V]	Pressure [mBar]	Results
120	2000	185	Good
220	2000	163	Good
320	2000	163	Good

Table X: Parameters used to encapsulate W/O emulsion (inner nozzle: 150 µm, outer nozzle: 400µm; 2 % (w/V) sodium alginate; 1 M $CaCl_2$ * $2H_2O$)

Frequency [Hz]	Electrode [V]	Pressure [mBar]	Results	Flow rate [mL/min] of shell solution	Flow rate [mL/min] of core solution
370	2000	245	Could be better	7.5	0.9
170	2000	245	Could be better	7.5	0.9
420	1500	245	Good	7.5	0.9
420	**2000**	**245**	**Very good**	**7.5**	**0.9**

Table XI: Parameters used to encapsulate W/O emulsion (inner nozzle: 200 µm, outer nozzle: 300 µm; 2 % (w/V) sodium alginate; 1 M CaCl2 * 2H2O)

Frequency [Hz]	Electrode [V]	Pressure [mBar]	Results
420	**2500**	**390**	**Very good**

Time in hardening solution

We had to find optimal time of hardening MCs. If beads are not kept long enough in hardening solution, the gelation may not be complete and if beads are left in hardening solution for too long, shell shrinks, which may lead to premature release of active ingredient.

MCs were hardened for at least 30 min to ensure complete gelation and then washed by distilled water and filtered using a porous membrane to remove any un-reacted components.

4.2. MICROCAPSULES PROPERTIES

4.2.1. SIZE DISTRIBUTION

The size distribution is not uniform for all MCs (Table XII). Samples of MCs are polydisperse mixture of powders; therefore size distribution is not normal, but log-normal. In the case of log-normal distribution, data on the abscissa are logarithmated. It is clear that MCs approximately follow log-normal distribution (Figures 18, 20, 22). The size of MCs loaded with RSV ranges from 401.05 – 687.56 µm, with an average of 529.93 µm (Figure 17), MCs loaded with beta-carotene ranges from 394.79 – 563.39 µm, with an average of 473.81 µm (Figure 19), whereas the particle size of MCs loaded with W/O emulsion ranges from 318.10 – 655.55 µm, with an average of 489.24 µm (Figure 21).

Table XII: Measured microcapsules size distributions are reported as volume mean and $d_{0.1}$, $d_{0.9}$ and $d_{0.5}$ µm values corresponding to the particle percentages below the stated number. Value of span corresponds to the ratio of the largest and smallest sizes. The span measures the width of the principle size distribution. A small span value indicates a narrow particle size distribution.

	Resveratrol MC	Beta-carotene MC	W/O emulsion MC
Volume mean D [4,3] [µm]	532.72	469.52	488.37
D (v 0.1) [µm]	401.05	394.79	318.10
D (v 0.9) [µm]	687.56	563.39	655.55
D (v 0.5) [µm]	**529.93**	**473.81**	**489.24**
Span	$\mathbf{5.406 * 10^{-1}}$	$\mathbf{3.558 * 10^{-1}}$	$\mathbf{6.898 * 10^{-1}}$

By calculating span values, we determined that the MCs loaded with W/O emulsion have the widest size distribution, while the MCs loaded with beta-carotene have the narrowest. The higher average diameter was obtained for MCs loaded with RSV and the lowest for MCs loaded with beta-carotene.

According to the literature data, the bead size is controlled by parameters, such as nozzle size, vibration frequency, flow rate, and physical properties of the encapsulated mixture (8).

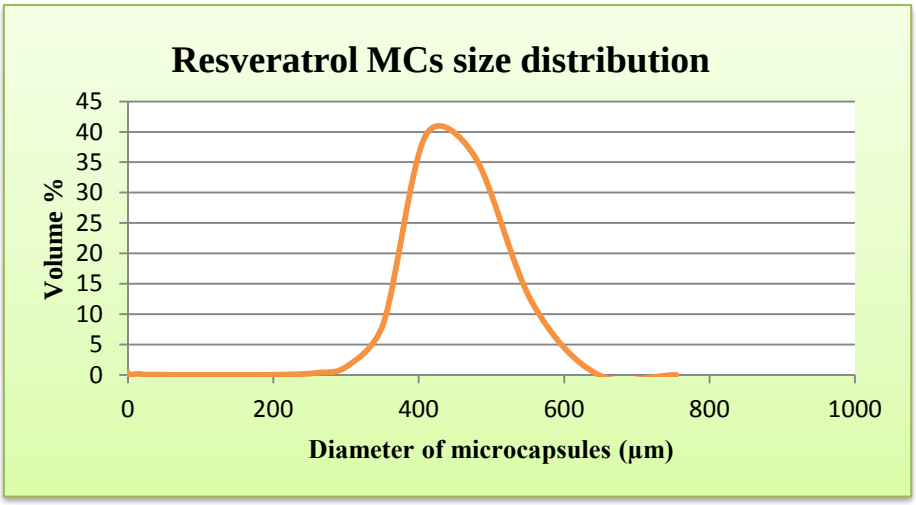


Figure 17: Resveratrol microcapsules size distribution.

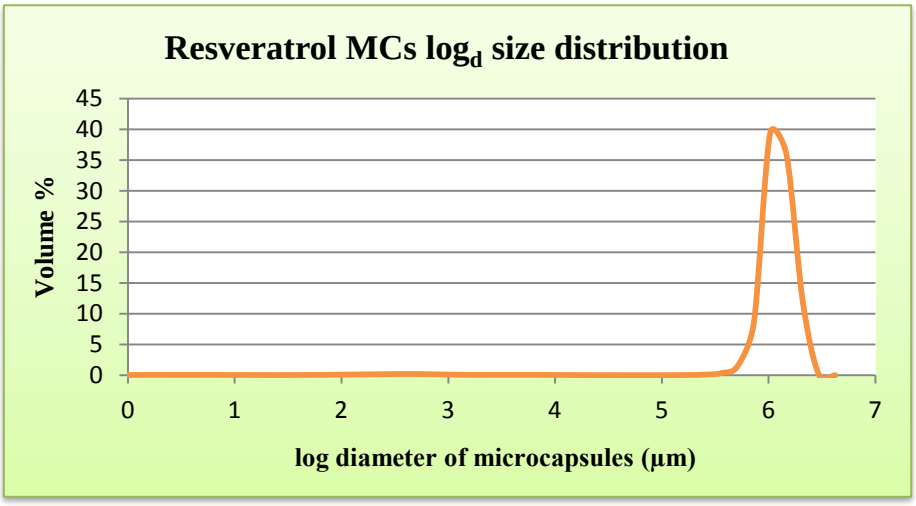


Figure 18: Resveratrol microcapsules $\log_d$ size distribution.

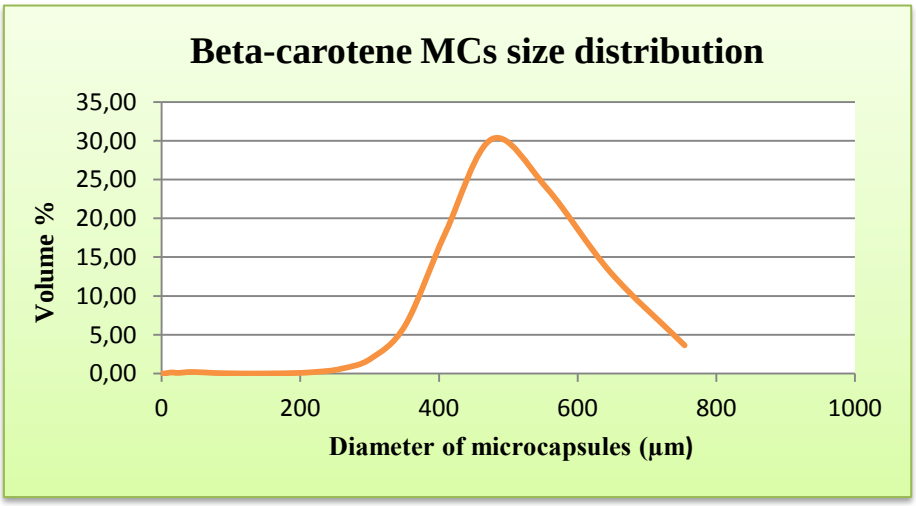


Figure 19: Beta-carotene microcapsules size distribution.

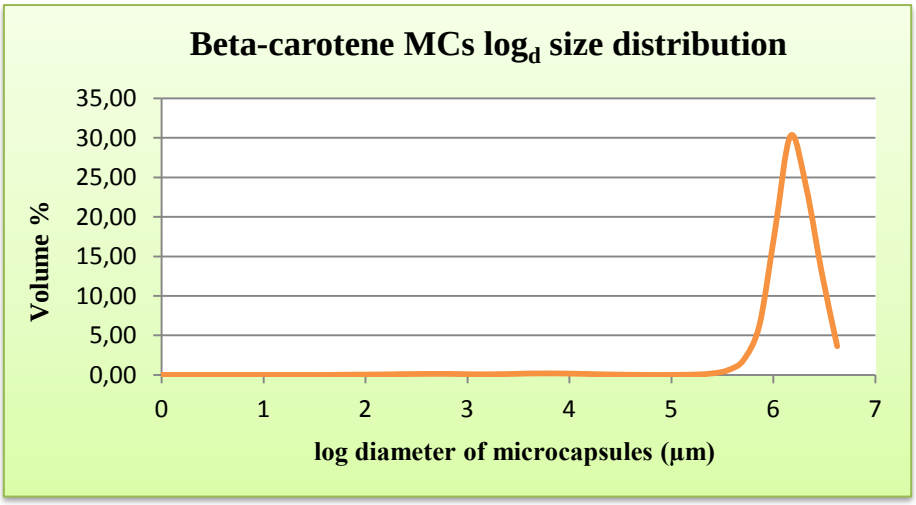


Figure 20: Beta-carotene microcapsules log$_d$ size distribution.

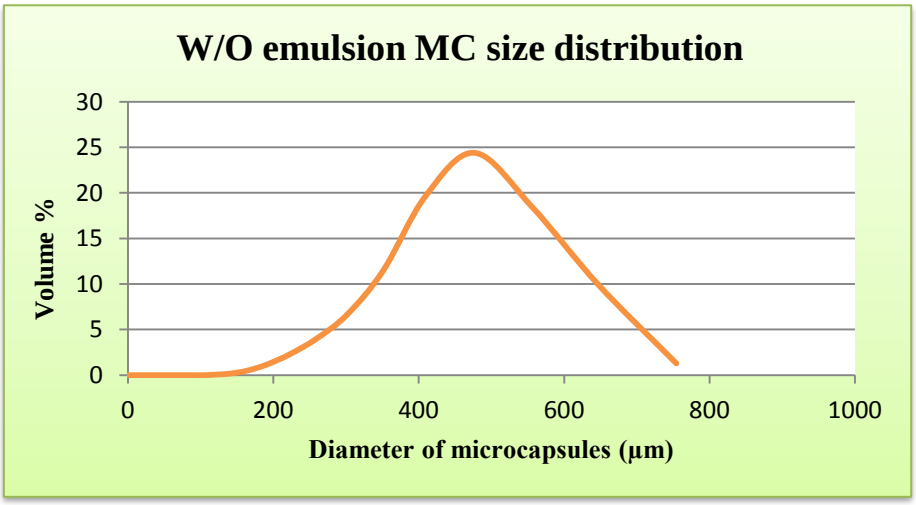


Figure 21: W/O emulsion microcapsules size distribution.

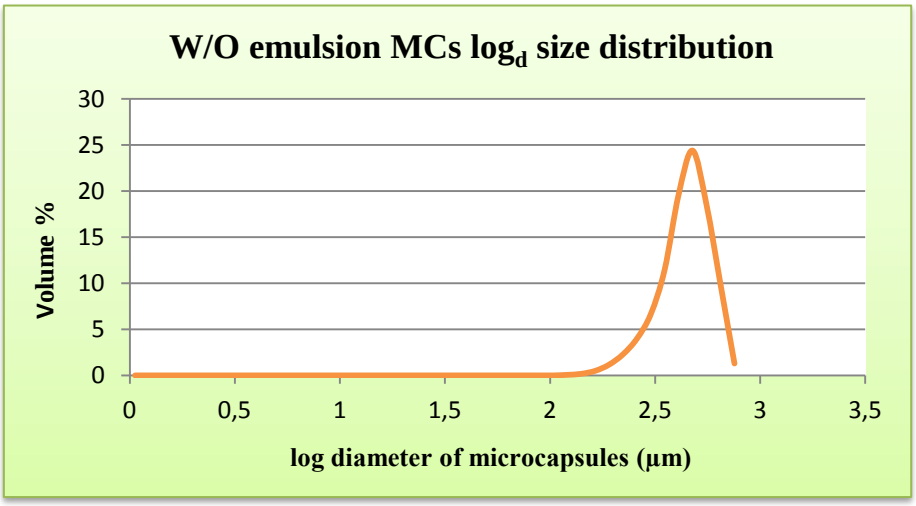


Figure 22: W/O emulsion microcapsules log$_d$ size distribution.

4.2.2. MORPHOLOGY OF MICROCAPSULES

MCs loaded with solution of RSV in oil, solution of beta-carotene in oil and W/O emulsion, were prepared under previously determined optimal conditions and studied in the terms of surface morphology, as showed in figure 23.

Microscope images of MCs in figure 23 show that MCs are mononuclear and most of them are in spherical form.

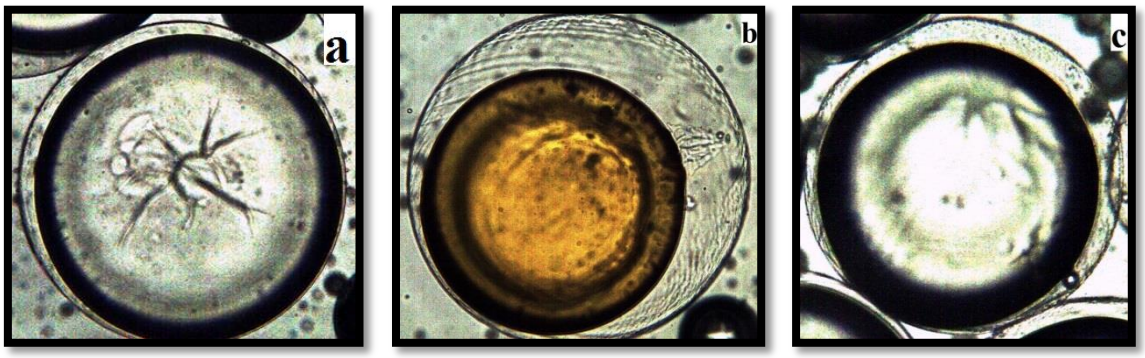


Figure 23: Images taken with light microscope at 200-fold magnification. (a) Microcapsule loaded with resveratrol; (b) microcapsule loaded with beta-carotene; and (c) microcapsule loaded with W/O emulsion.

4.2.3. SHELL THICKNESS

Average shell thicknesses of MCs loaded with RSV, beta-carotene, and W/O emulsion were 30 µm, 45 µm, and 41.25 µm, respectively (Table XIII). In all experiments we produced MCs with thin shells, which can be a very important characteristic as it has the ability to significantly affect the release of the compounds from MCs (8).

4.2.4. ENCAPSULATION DEGREE [%]

Average encapsulation degree of MCs loaded with RSV, beta-carotene, and W/O emulsion was 69 %, 53.25 %, and 57.61 %, respectively (Table XIII). Encapsulation degree of produced MCs should be higher, because the highest the encapsulation degree, the thinner the shell, which affects the release of the compounds from MCs, as discussed above. We should use a bigger inner nozzle according to outer nozzle or increase the flow rate of core material.

Table XIII: For determination of encapsulation degree and shell thickness of microcapsules, average diameters and volumes of microcapsules and cores were measured with help of light microscope.

	Resveratrol MC	Beta-carotene MC	W/O emulsion MC
Average MC Ø [µm]	518.75	475	491.25
Average MC volume [µm^3]	73096749.85	56115062.53	62077302.09
Average core Ø [µm]	458.75	385	408.75
Average core volume [µm^3]	50553968.62	29880014.98	35760517.40
Encapsulation degree [%]	**69**	**53.25**	**57.61**
Shell thickness [µm]	**30**	**45**	**41.25**

4.2.5. ENCAPSULATION EFFICIENCY

Calibration line

For the study of encapsulation efficiency we prepared a calibration line of beta-carotene in the concentration range of 6.25 – 100 mg/L. Corresponding absorbance was measured by UV-Vis spectrophotometer at absorption maximum of beta carotene in oil solution (λ = 461 nm) (Figure 24).

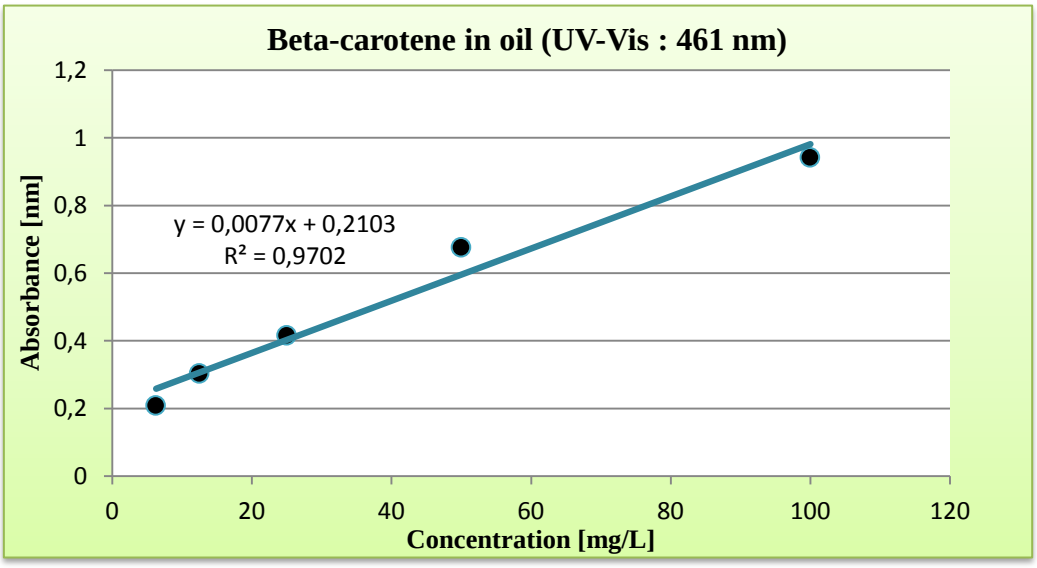


Figure 24: Calibration line (absorbance of beta-carotene in almond oil at λ = 461 nm as a function of concentration).

The value of R^2 for calibration line is close to 1, which means that the dispersion of the data is suitable small. Deviations of the measured data can be attributed to errors in pipetting and measurement.

Encapsulation efficiency

Absorbance of beta-carotene in almond oil after extraction from MCs was measured at 461 nm by UV-Vis spectrophotometer. By means of calibration line and equation *9*, we determined that the incorporation of beta-carotene in almond oil after microencapsulation is 79 % according to concentration before microencapsulation (Figure 25). The result is sufficient; however encapsulation efficiency under ideal conditions can be reach up to 90 %.

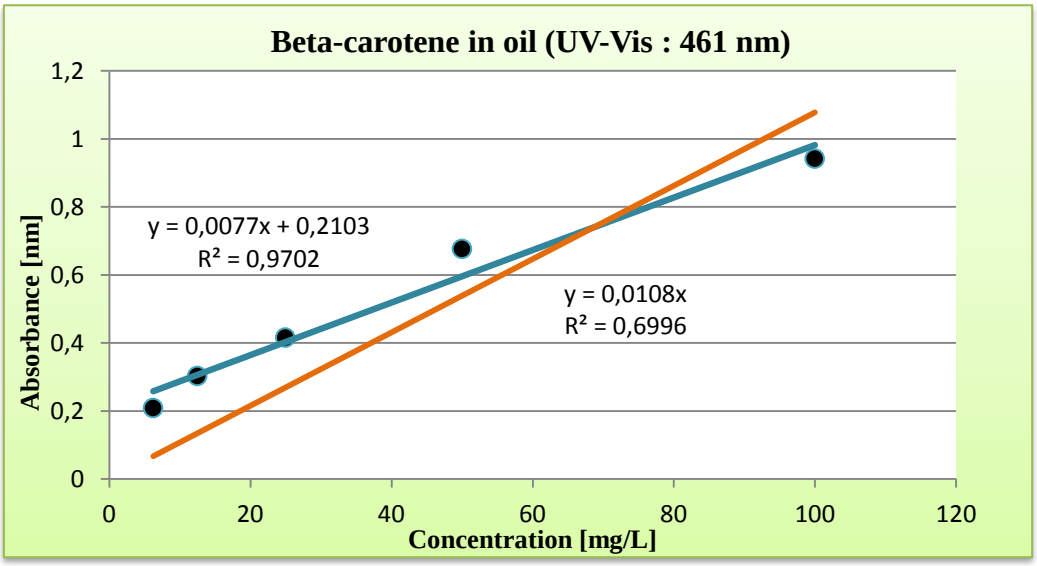


Figure 25: Measured absorbance of beta-carotene in almond oil after microencapsulation and determination of concentration of beta-carotene in almond oil after microencapsulation, corresponding to calibration line.

The literature data reveal that the encapsulation efficiency is mainly dependent on the mannuronic/guluronic (M/G) ratio of alginate and content of water in bead. In general, the high-M beads show higher encapsulation efficiency than the high-G beads, due to swelling to a larger volume (35). We cannot know the exact M/G ratio of our alginate, which mainly depends on the species of origin, location of alginate in the plant and the time of year in which harvesting was performed (8). It was found that the beads with lower content of water have higher encapsulation efficiency. This applies to both types of alginate (35). It

has been also documented that incorporation of surfactant in hardening solution improves the encapsulation efficiency due to reducing the interfacial tension, leading to formation of small capsules (36).

5. CONCLUSION

In the present diploma thesis we produced microcapsules by vibration nozzle method, which proved to be an efficient method for manufacturing microcapsules.

Initially, optimal process parameters were determined. Physical parameters, such as viscosity of alginate solution and concentration of hardening solution were determinated empirically. 2 % (w/V) alginate solution and 1 M hardening solution were found to be appropriate, as produced microcapsules were well-formed, mononuclear and most of them spherical as judged from observations with light microscope. Alginate solution of too low viscosity and hardening solution of too low concentration can cause faster, easier and maybe premature release of active ingredient, but too viscous alginate solution and too concentrated hardening solution can prevent release, as the shell is too rigid and compact.

For each combination of outer and inner nozzle diameters or change in alginate or core solution viscosity, the optimal process parameters such as vibration frequency, electrode tension and flow rates, would have to be determined once again in the light of encapsulator stroboscope. It is very important that one understand how each parameter works and influences the properties and the quality of produced microcapsules.

The final and very important parameter in producing microcapsules is how long to leave them in hardening solution. Our experiments showed that after one hour of hardening, shrinking of shell was caused and consequently release of core solution. However, microcapsules have to stay in hardening solution for at least 30 minutes, to ensure complete gelation.

Morphology tests under light microscope showed that by vibration nozzle method and appropriate process parameters, well-formed, mononuclear and mostly spherical microcapsules are manufactured. Additional observation under light microscope also showed that microcapsules with controlled shell thickness and high encapsulation degree can be produced. Laser diffraction showed that microcapsules with approximately narrow size distribution are produced.

As one of the most important results of encapsulating liquid formulations with antioxidants by vibrational nozzle method, encapsulation efficiency by UV-Vis spectrophotometer was determined as well. We produced microcapsules with 79% encapsulation efficiency, which is an acceptable result, although under ideal conditions, encapsulation efficiency up to 90% can be achieved. For higher encapsulation efficiency, we would need to test different batches of sodium alginate, as encapsulation efficiency is

mainly influenced by alginate M/G ratio, which depends on the species of the origin, location of alginate in the plant and the time of year in which harvesting was performed.

To sum up, our experiments showed that microencapsulation by vibration nozzle method offers a broad range of new applications in cosmetic industry, making production of mononuclear liquid core microcapsules loaded with formulations of antioxidants easy and reproducible. Therefore, antioxidants are protected against UV light, heat and oxidation and their release can be controlled and targeted.

Even though substantial progress has been made in the field of microencapsulation, many challenges still remain, e.g. selection of appropriate core and shell materials and adjusting process parameters. The advantages of vibration nozzle method are automation, rapidity and the ability of avoiding organic solvents use.

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