ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

ENCAPSULATION OF FOOD FLAVORS VIA COACERVATION METHOD

M.Sc. THESIS

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Department of Advanced Technologies

Molecular Biology–Genetics & Biotechnology Programme

JUNE 2012

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GIDA AROMALARININ KOASERVASYON YÖNTEMİ İLE ENKAPSÜLE EDİLMESİ

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To my family,

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ABBREVIATIONS

G	**:** Gelatin
GA	**:** Gum Arabic
GTA	**:** Glutaraldehyde
CLSM	**:** Confocal Laser Scanning Microscopy
GC	**:** Gas Chromatography
NR	**:** Nile Red
NHN	**:** Ninhydrin
DMSO	**:** Dimethylsulfoxide
OD	**:** Optical Density
dH20	**:** Distilled Water
NaOH	**:** Sodium Hydroxide
HCl	**:** Hidrocloric Acid
AG	**:** Arabinogalactan
AGP	**:** Arabinogalactan Protein
GP	**:** Glycoprotein

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ENCAPSULATION OF FOOD FLAVORS VIA COACERVATION METHOD

SUMMARY

Flavors are used in many areas, such as food, cosmetic, pharmaceutical and detergent industries to increase taste and/or odor. Production of these compounds by environmental-friendly biological processes instead of conventional chemical synthesis and design of new delivery system for these high value added compounds to preserve their properties are upmost importance for customer satisfaction. These highly valuable compounds are very expensive and they lose their effect beginning from the production step due to their volatile and oxidation labile nature. The decreases in properties continue during the transfer and storage of the product. To prevent this problem, encapsulation of flavors into different materials (e.g. gelatin, gum arabic, whey proteins, pectin, etc) is a well known method.

In this study, the aim is to determine the potential of coacervation method and the usage of a natural crosslinker (i.e. genipin) in the efficient encapsulation of food flavors. Investigation of the effect of production conditions to microcapsule properties was also studied. As a model flavor, orange oil was encapsulated into different ratio of gum arabic/gelatin mixtures. Total amount of gelatin and gum arabic were kept constant but their ratio were changed. Highest encapsulation yield was found at 3:2 (gelatin/gum arabic, w/w). When gelatin or gum arabic amounts were higher than this optimum condition, newly formed microcapsules were aggregated and gelleted. Apart from the ratio of wall materials, core/wall material ratio and stirring rates were studied. For this purpose corn oil was used as the core material instead of flavor agent for initial optimization. Prepared microcapsules were crosslinked by a natural agent, namely genipin, that is non-toxic unlike glutaraldehyde. Microcapsules were cosslinked with different concentrations of genipin solutions (2-30 mM in dH_20) and dried by lyophilization. Preparation conditions and different crosslinking agent concentrations were studied for their effect on coacervation properties. Crosslinking could be visualized by the blue color

generation after genipin addition. Optimum ratio was found to be 2:3 (w:w) for gum arabic/gelatin and with high genipin concentrations (30 mM), blue color was obtained after an overnight incubation at room temperature while at low concentrations (2 mM), incubation time should be extended to 3 days. In addition to that, ninhydrin assay was applied to find optimum genipin concentration for gelatin/gum arabic mixture. Encapsulation yield was determined to be 57.8 % for corn oil. Moreover, microcapsules were visualized in fluorescence microscopy using nile red as lipid staining dye. Size distribution for optimum conditions were found to be 250-350 μm.

In conclusion, genipin was proved to be a suitable crosslinker for the encapsulation of food ingredients. The encapsulation procedure should, however, be optimized to increase the yield for these volatile compounds.

GIDA AROMALARININ KOASERVASYON YÖNTEMİ İLE ENKAPSÜLE EDİLMESİ

ÖZET

Aromalar, gıda ve kozmetik endüstrisi gibi birçok alanda koku ve/veya tat duyusunu arttırmak amacıyla kullanılmaktadır. Genellikle kimyasal teknoloji ile üretilen bu bileşiklerin daha çevre dostu biyolojik süreçlerle üretilmesi ve üretilen katma değeri yüksek bu ürünlerin özelliklerini kaybetmeden tüketiciye ulaştırılması büyük önem taşımaktadır. Aromalar, kolay buharlaşmaları ve çevresel koşullardan çabuk etkilenerek özelliklerini kaybetmeleri nedeniyle daha ürünlerin hazırlanması aşamasında etkilerini yitirmeye başlamaktadır. Ürünlerin transferi ve tüketiminden önce geçen zaman içinde ise bu etki kaybı sürmektedir. Bu problemin giderilmesinde aromaların çeşitli malzemeler (polisakkarid, protein, vb) içine hapsedilmesi (enkapsülasyonu) çok bilinen bir yöntemdir. Enkapsülasyon endüstriyel olarak önemli bir süreçtir. Sıvıların, gazların veya katıların bir duvar ya da ince bir koruyucu tabaka ile etrafı sarılarak hapsedilen aktif bileşiklerin kimyasal bozunmaları ve buharlaşmaları engellenir. Ayrıca, aktif bileşiklerin salımı belli koşullarda ve uzun süre kontrol altında tutulabilir. Gıda, biyoteknoloji, kozmetik, ilaç ve tarım gibi bir çok endüstride bir çok değişik metodu sıklıkla kullanılmaktadır. Bunlar fiziksel, kimyasal ve fizikokimyasal olmak üzere sınıflandırılabilir. Koaservasyon methodu da bu metotlardan bir tanesidir ve iki birbirine karışmayan koloid sisteminde faz ayrılması olarak tanımlanır. Kompleks koaservasyon metodunda suda çözünebilen iki farkı yüke sahip duvar malzemeleri kullanılır ve bir polimerin eksi yükü ile diğerinin pozitif yükünün nötralizasyonu ile polimer bakımından zengin fazın ayrılmasına neden olur. Duvar malzemeleri bu bakımdan oldukça önemli bir role sahiptir. Seçilen duvar malzemesinin kapsüle edilecek malzemeyle herhangi bir etkileşiminin olmamasına dikkat edilmeli ve bununla birlikte suda çözünebilir, biyouyumlu ve biyoparçalanabilir özelliklere sahip olmalıdır. Jelatin ve arap zamkı kompleks koaservasyon yönteminde sıklıkla

kullanılan iki polimerdir. Jelatin protein yapıda olup pozitif yüke sahiptir, arap zamkı ise polisakkarid olup negatif yüke sahiptir. Enkapsülasyon prosedürü süresince oluşan mikroküreler oldukça düşük mekanik kuvvete ve termal kararlılığa sahiptir. Bu durumu engellemek için mikroküreler çapraz bağlayıcı olarak isimlendirilen bifonksiyonel kimyasal bileşikler kullanılarak daha sağlam yapılara dönüştürülür. Bu yüzden duvar malzemesinin seçilmesi kadar kullanılacak olan çapraz bağlayıcıların seçimi de bir o kadar önemlidir. Glutaraldehit sıklıkla kullanılan ucuz, etkili bir şekilde kısa sürede çapraz bağ oluşturan ama toksik bir çapraz bağlayıcıdır. Genipin ise doğal kaynaklardan elde edilen, toksik olmayan, biyouyumluluğu yüksek bir çapraz bağlayıcıdır.

Bu çalışmada, doğal bir çapraz bağlayıcı (yani genipin) kullanılarak gıda aromalarının verimli bir şekilde hapsedilmesi ve koaservasyon yönteminin potansiyel kullanımının belirlenmesi amaçlanmıştır. Ayrıca, üretim koşullarının mikrokürelerin oluşumu üzerine etkileri de incelenmiştir. Portakal yağı aroma, değişik oranlardaki jelatin/arap zamkı ise duvar malzemesi olarak seçilmiştir. İlk optimizasyon çalışmaları için mısır yağı, portakal yağı yerine kullanılmıştır. Duvar malzemelerin oranı dışında, aroma/duvar malzemesi oranı ve emülsiyon oluşturma aşamasında da farklı karıştırma hızları denenmiştir. Daha sonra hazırlanan mikrokapsüller, glutaraldehidin aksine doğal ve toksik olmayan genipin ile çapraz bağlanmıştır. Genipinin farklı konsantrasyonlarda (2-30 mM) ve farklı reaksiyon sürelerindeki (20-120 saat) etkisi incelenmiştir. Oluşan mikrokapsüller toplandıktan sonra dondurarak kurutma ile kurutulmuştur. Genipin eklendikten sonra oluşan mavi renk değişimi görsel olarak da izlenmiştir. Buna ek olarak optimum genipin konsantrasyonunu belirleyebilmek için çapraz bağlanmamış serbest amino gruplarının tayininde kullanılan ninhidrin deneyi uygulanmıştır. Aroma enkapsülasyon verimi döner buharlaştırıcı kullanarak tespit edilmiştir. Buna ek olarak, floresan boyası olarak yağda çözünen nil kırmızısı kullanılarak taramalı konfokal lazer mikroskobu ile de mikrokapsüller görüntülenmiştir.

Toplam jelatin ve arap zamkı miktarı sabit olmak üzere oranlar değiştirildi. En yüksek mikrokapsül verimi jelatin/arap zamkı oranının 3:2 (ağırlıkça) olduğu örnekte gözlemlendi. Jelatin ya da arap zamkı oranının optimum koşullardan yüksek olduğu örneklerde (konsantrasyonlarının sırasıyla % 40 ve % 50 arttırılmasıyla) yeni oluşan mikrokapsüllerin kümelendiği ve jelleşme oluştuğu gözlemlendi. Genipin ile çapraz

bağlama deneyleri sonucunda yüksek konsantrasyonlarda (10-30 mM) kullanılan genipin ile oda sıcaklığında bir gece bekletilen mikroküre lerde mavi renk oluşumu gerçekleştiği fakat düşük konsantrasyonlarda (2-5 mM) genipin kullanıldığına reaksiyon süresinin ve mavi renk oluşumunun 3 güne kadar uzadığı görüldü. Ancak, ninhidrin deneyleri sonucunda farklı genipin konsantrasyonları ile belirgin bir fark oluşmadığı görüldü ve aynı zamanda verim hesaplamalarına göre glutaraldehit ile çapraz bağlanan mikrokapsüllerin veriminin genipin ile çapraz bağlananlardan daha yüksek olduğu bulundu. Optimum koşullar altında mısır yağının verimi, glutaraldehit ile çapraz bağlanan mikrokapsüllerde % 81,6 iken genipin ile çağraz bağlanan mikrokapsüllerde % 57,8 olarak hesaplandı. Portakal yağı kullanılarak yapılan deney sonuçlarında verimin oldukça düşük olduğu görüldü (% 4,5) ve hatta bazı deneyler sonucunda mikrokapsül oluşmadığı gözlemlendi. Aynı zamanda, prosedür boyunca çevresel faktörlerin de (sıcaklık gibi) enkapsülasyon verimine ve mikrokapsüllerin oluşumu üzerine etkili olduğu görüldü. Mikrokapsüller nil kırmızısı kullanılarak floresan mikroskobunda görüntülendi ve optimum koşullarda, mikrokapsül boyutlarının 250-300 μm olduğu görüldü. Sonuç olarak, çapraz bağlayıcı olarak kullanılan genipinin enkapsülasyon işlemi için uygun olduğu ancak veriminin düşük olduğu görüldü ve enkapsülasyon koşullarının uçucu bileşikler (portakal yağı) için daha iyi optimize edilmesi gerektiğine karar verildi. Daha sonraki çalışmalarda farklı duvar malzemeleri kullanılabilir ve farklı pH değerlerinde genipinin reaksiyonu gerçekleştirilebilir.

1. INTRODUCTION

1.1 Purpose of Thesis

Encapsulation of flavors into different materials (e.g. gelatin, gum arabic, whey proteins, pectin, etc.) is a well known method. In this study, the potential of coacervation method to encapsulate food flavor into gelatin/gum arabic mixture and the use of genipin as a crosslinker were studied. Genipin was selected as cross linker because it is a natural, non-toxic agent unlike glutaraldehyde. Orange oil was chosen as model flavor but corn oil was used instead of flavor agents in the first optimization studies. Microcapsules were prepared with diverse preparation conditions such as different gelatin/ gum arabic ratios, different core material/wall materials ratios and with different stirring rates. Furthermore, prepared microcapsules cross linked with different concentrations of genipin solutions and dried by lyophilization.

1.2 Encapsulation

Encapsulation or microencapsulation is a method to entrap one material (active agent) or a combination of materials within another material (wall material) or in other words entrapped active material within another carrier material or system [1-3]. The encapsulated substance can be called the core, fill, active, internal and the encapsulating material is often called the coating, membrane, shell, capsule, carrier material, wall material, external phase, or matrix [3]. Encapsulation aims to protect stability of the bioactive compounds during processing and storage and to prevent undesirable interactions with food matrix. Mainly, bioactive food compounds are characterized by rapid inactivation [4]. So, this method is an industrially important process for physically coating liquids, solids, or gases with in thin, protective layer or wall material to prevent their loss by volatilization and to protect them against chemical disruption [5] and also the release of their contents could be kept at controlled rates over extended periods and under specific conditions. Usually produced particles' diameters varies between a few nm to a few mm [3]. The

improvement of microencapsulation products started in 1950s in the research of pressure-sensitive coatings for the manufacture of carbonless copying paper [1]. Encapsulation technology is now well developed and accepted and is a technique that is widely used to encapsulate food flavor agents**,** drugs, proteins**,** antigens**,** cells**,** and enzyme, etc within the pharmaceutical, chemical, cosmetic, foods and printing industries [1,6]. Materials used in the design of protective shell of microcapsules can be selected from a wide variety of polymers such as carbohydrates, cellulose derivatives, gums, lipids and proteins, depending on the core material and desired characteristics of the microcapsule. In addition, selected materials must be food-grade, biodegradable and they also should be able to form a barrier between the internal phase and its surroundings [3,5]. Depending on the physicochemical properties of the core, wall material, the wall material composition, and the used microencapsulation technique, different morphologies given in Figure 1.1 could be obtained: simple microcapsules coated by an uniform thickness; particle containing an irregular shape core; more than one core particles embedded in a continuous matrix of wall material; several distinctive cores within the same capsule and multi walled microcapsules [4].

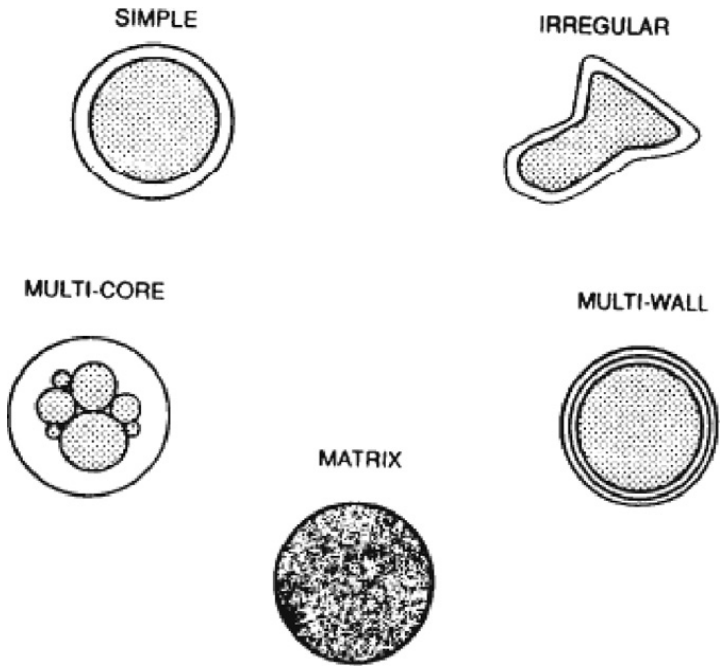


Figure 1.1 : Morphology of different types of microcapsules [4].

Encapsulation techniques can be separated into three classes: chemical processes like molecular inclusion or interfacial polymerization, physicochemical techniques like coacervation and liposome encapsulation and physical processes like spray drying,

freeze drying, spray chilling/cooling, co-crystallization, extrusion or fluidized bed coating [4,7].

1.2.1 Encapsulation methods

As mentioned in section 1.2, there are various techniques for microencapsulation. The most common methods of microencapsulation or encapsulation are extrusion, interfacial polymerization, solvent evaporation, phase separation (coacervation), and spray drying [6]. Since active compounds to be encapsulated are frequently in a liquid form, many technologies are based on drying, for example, spray- or freeze-drying, can be used in oil-in-water emulsion systems to produce powder [3]. Similarly, different techniques have been successfully used for preparing flavor loaded microcapsules. Briefly, spray-drying is one of the oldest and common encapsulation techniques and also it is the cheapest method for producing microcapsules [7]. It has been extensively used in the food industry. Generally, the prepared particles are small but the size distribution of particles is very wide. Interfacial polymerization always involves the usage of many organic solvents and non-biocompatible wall materials. Solvent evaporation also involves the usage of relatively more organic solvents [6]. Another frequent technique is emulsification. It is employed in case of water soluble food active agents and there are two combinations of emulsions, one of them is water/oil emulsions and the other one is oil/water emulsions [3]. One of the other techniques in use for microencapsulation is freeze-drying. In the freeze-drying process, the solvent is removed from a frozen solution by vacuum sublimation, maintaining the drying chamber at low temperatures. Freeze drying appears as one of the most suitable methods for dehydration of almost all heat-sensitive materials and flavor, due to the lower operating temperatures, slow drying rate and vacuum conditions [7]. In a similar way, vacuum-drying could be applied as the drying process, and its advantage is that it is faster and cheaper than freeze-drying, because it operates at a temperature above the freezing point of the solvent [7]. The major disadvantages of freeze-drying are the high energy input and long processing time. In addition, during processing a barrier with an open porous structure between the active agent and its surroundings is formed; this high-porous wall offers poor protection when prolonged release of an active is required [3]. Extrusion is another method, which includes the forcing of a solution or suspension of core material and wall material through a pipette, syringe, a

coaxial needle or a spraying nozzle [6,7]. Finally, spray-chilling or spray-cooling are technologies used in encapsulation to produce lipid-coated active agents [3].

1.2.1.1 Coacervation method

Coacervation is a one of the microencapsulation method that is defined as the separation into two liquid phases in colloidal systems [8]. The basic idea behind microencapsulation by coacervation is phase separation; there are two phases: the first one is a rich solvent phase with very small amounts of biopolymer(s) and the other one is a rich biopolymer(s) phase [9] and "coacervate" that is more concentrated in colloid component [8].

Coacervation is divided into two categories: simple coacervation and complex coacervation. Simple coacervation usually interests only with one colloid solute and includes the addition of a strongly hydrophilic compound to a less hydrophilic colloidal dispersion. It generates two layers: one rich in colloid droplets, and the other deficient in such droplets. On the other hand, complex coacervation involves more than one colloid and caused by the interaction of two oppositely charged colloids [8] between a poly-anion and a poly-cation, both water-soluble [6]. In order to generate complex coacervation, various macromolecular systems have been used. Gelatin/gum acacia system is one of them and probably most widely studied system [10]. Other systems are also used for coacervation like heparin/gelatin, gelatin/carboxymethyl cellulose, polilactids and co-polimers of lactic and glycolic acid, polivinil alcohol, hydroxyl propylmethyl cellulose phthalate, plant proteins, polyurethane [11]. Properties of coacervate layer, that is, shell material (physicochemical, thickness, microstructure, etc.) are key points to determine behavior of microcapsules (stability, redispersibility, microencapsulation efficiency, etc). Hence, choice of the wall material and interaction among its components are other critical issues for successful microencapsulation by coacervation [11].

Tiebackx was the first researcher who reported on the coacervation [8], but Bungenberg de Jong and Kruyt [8] were the first to studied the phenomenon systematically on the gum arabic-gelatin system. The work was followed by the development of a first theoretical model by Overbeek and Voorn [8]. Subsequent theoretical models were developped by Veis et al., Nakajima and Sato, Tainaka [8]. Generally, complex coacervation refers to the separation of an oppositely charged

polyion mixture (polyelectrolytes) into two distinct phases [12] by media modifications [10,13]. The complex coacervation was significantly affected by the pH of the solution, since pH determines the charge density on ampholytes and may even induce structural transitions of proteins and polysaccharides [13]. Briefly, gelatin, which is cationic at pH values below its isoelectric point, may interact with a variety of natural and synthetic anionic water soluble polymers, such as gum arabic, to form complex coacervates suitable for encapsulation [6]. Neutralization of the positive charge of one of the polymers by the negative charge of the other polymer causes phase separation of the polymer-rich phase [10]. Principle of complex coacervation method was shown in the Figure 1.2.

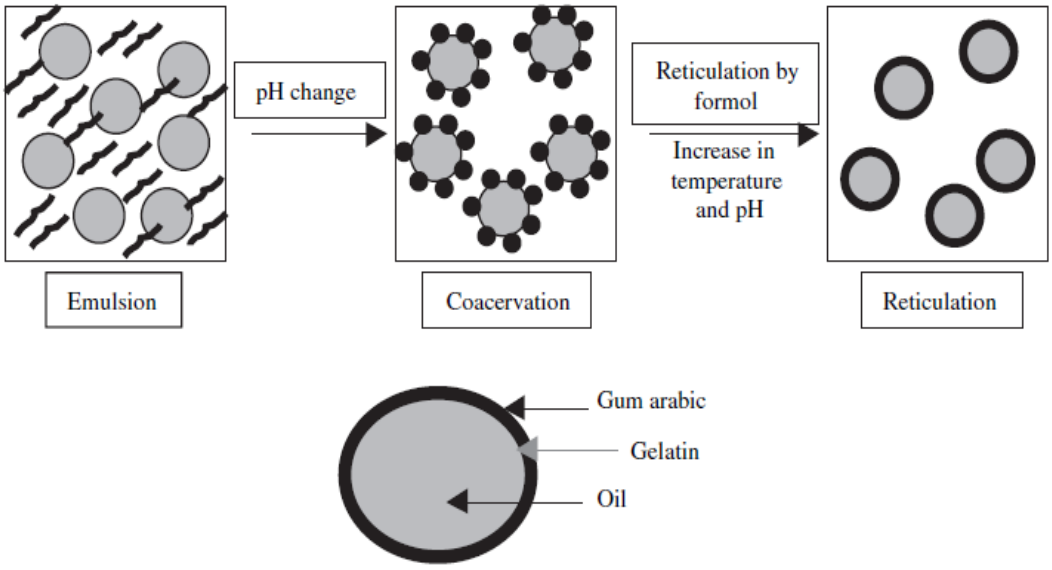


Figure 1.2 : Principle of the complex coacervation method [1].

Microcapsules produced by coacervation were reported to have an excellent controlled release characteristics and heat resistance properties [13]. The gelatin/gum arabic system has been successfully used in the production of carbonless paper, scent strips, fragrance samplers and flavor ingredients [13]. Although surfactants are often added to a system to improve wettability of the coacervate droplets, their concentration should be carefully optimized. Inhibition of coacervation due to high concentrations of surfactants and disorders of microencapsulation because of high hydrophile lipophile balance (HLB) values have been reported [12]. The control release property of coacervate microcapsules is determined by the structure, the particle size, the loading, the cross-linking degree and dispersing medium. The structure of coacervate microcapsules is considerably affected by homogenization

rate during emulsification process. Mononuclear microcapsules prepared with a lower homogenization rate, release the internal core material more rapidly than multinuclear microcapsules produced by high homogenization rate. Smaller microcapsules possess bigger surface area and thinner membrane, therefore the core material is easier to diffuse into the release medium through microcapsules membrane [14].

1.2.2 Encapsulation of food flavor compounds

There are number of techniques available for encapsulation of food compounds [3]. Encapsulation is an industrially crucial process. Encapsulation of a food compound such as reactive and volatile oils and flavors into a protective wall material led to protection of them against evaporation, chemical reactions (such as flavor-flavor interactions, light induced reactions, oxidation) or migration in the food [3]. Encapsulation decreases the evaporation and degradation rate of volatile actives, such as flavor, which usually contains mixture of volatile and odorous organic molecules [1]. According to public surveys among consumers one of the most important properties of foods is the taste and the taste based on flavor activities the primary basis upon which food is selected and reselected. Factors that are effective for flavor delivery in the mouth include the composition and microstructure of the product, dilution and mixing with saliva, changes in temperature, flavor concentration and the occurrence of reversible/irreversible flavor binding. Moreover, mass transfer of flavor in the mouth is affected by the gas and saliva flow rates, the degree of agitation, and the temperature, which are all affected by the food structure and composition [15]. Furthermore, flavors can be among the most valuable ingredients in any food formula. Even small amounts of some flavor compounds can be expensive, and they are usually delicate and volatile, preserving them is a challange for food manufacturers [1]. Another reason for using encapsulation is to prevent reaction with other components in food products such as oxygen or water, e.g. in case of essential oils [3]. For encapsulation of flavor compounds, the carrier material must have no reactivity with the core material; should be present in a form that is easy to handle, i.e. with low viscosity at high concentration, allow a complete elimination of solvent in any processes; give the maximum protection of the active ingredient against external factors; ensure good emulsion–stabilization properties and effective redispersion behavior in order to release the flavors at the desired time and

place [5]. Potential formulations in encapsulation include liquid forms (emulsions, micelles, liquid solutions etc.), semi-liquid forms (gels, liposomes etc.), and solid forms (microcapsules or microcomposites) [7].

Flavor encapsulation can be achieved by a plenty of methods like spray-drying, spray-chilling or -cooling, freeze drying, coacervation and others [3]. Proper microencapsulation method relies on the end use of the product and the procedure conditions involved in the manufacturing process. Spray drying is the most common technique to produce flavor powders from food flavor emulsion, but coacervation is a unique and promising microencapsulation technology [1]. Most part of the literature available on complex coacervation mention about pharmaceutical applications. Encapsulation of flavor compounds, on the other hand, differs remarkably from the encapsulation of drugs, peptides or genes because of the different intrinsic physical properties of aroma compounds, including small molecular weight, high volatility and sensitivity to oxidation and degradation [16]. In general, microencapsulation is used to prevent unpleasant feelings during eating, such as bitter taste and astringency of polyphenols and other compounds that show high antioxidant activities and flavor releases from food before and after eating depending on the aroma features and physical state of the matrix [3].

1.3 Application of Encapsulation Method in Food Industry

Microencapsulation is an effective method and has a great potential in extensive application fields [17] and defined as the coating of a given material [17,18] for protection, isolation, or controlled release of the enclosed material [18]. It has attracted significant attention over the past two decades and has been currently applied in the food, biotechnology, and biomedical industries [18], as well as many other areas such as agriculture, cosmetic,catalysis, carbonless copying paper and so on [17]. In the food industry, encapsulation process can be applied for a lot of different applications. Encapsulation is useful to improve delivery of bioactive molecules (e.g. antioxidants, minerals, vitamins, phytosterols, lutein, fatty acids, lycopene) and living cells (e.g. probiotics) into foods. In recent years, the food industry concentrated its efforts to the production of functional foods which includes the addition of functional compounds in products. These compounds are usually highly sensitive to environmental, processing and/or gastrointestinal conditions.

Successful encapsulation of them is therefore important to control their flavor, color, texture or preservation properties since it slows down the degradation processes (e.g., oxidation or hydrolysis) or prevents degradation until the product is delivered at the desired sites. Thus, the bioactive component would be kept fully functional. Also, this technology may provide barriers between sensitive bioactive materials and the environment, and thus, to allow taste and aroma differentation, mask bad tasting or smelling, stabilize food ingredients or increase their bioavailability [3].

1.4 Wall Materials

Wall or carrier material plays a crucial role because it is responsible for the protection of the core material and for enabling a controlled release. This material must be selected depending on the specific core material to be coated and the desired characteristic of final microcapsules. Ideally, the wall material should be soluble in water, biodegradable, form low viscosity solutions, yield powders with specific properties (non-hygroscopic, non-porous, soluble, stable, etc.), have a low cost and be easy to dry and non reactive [7]. Different coating materials have been used in encapsulation techniques, including gums, starches, gelatins and polymers depending on the core material and desired characteristics of the microcapsule [5,7]. Moreover, for encapsulation of flavor compounds, the carrier material must have no reactivity with the core material [5]. Another important parameter is the charges of the polymers. This will be altered depending on the pH, the type and amount of colloid, the ratio of the two colloids (positive charges vs. negative charges) and their charge density.

1.4.1 Gelatin

Proteins are one of the extensively used materials in not only the food industry but also in other fields (e.g., drug and nutrient delivery) because of their superior properties like biodegradability, biocompatibility, nontoxicity, and non carcinogenicity [19-21]. In all hydrocolloids used in the food industry, gelatin has been pointed as special and unique, have multiple functions in numerous applications in various industries. It has been used as a fundamental material for microcapsules, sealants, tissue adhesives and carriers for controlled delivery systems [22], as a food ingredient (e.g., gelling and foaming agent), in the preparation of pharmaceutical

products (e.g., soft and hard capsule, microspheres), in the biomedical field (wound dressing and three-dimensional tissue regeneration) and in numerous non-food applications (e.g., photography) [23]. Furthermore, apart from being a water-soluble material with wall-forming ability gelatin has all the properties of an effective entrapping agent such high emulsifying activity and high stabilizing activity. Nevertheless, there are some challenges in the use of proteins as encapsulating agents like allergy and precipitation risk which could be observed when protein based microcapsules are added to food products and some religious and social (halal, kosher, vegetarian food choices) issues must be considered [4]. Also, the main limitation of gelatin is that it dissolves rather rapidly in aqueous environments [4] and this makes the use of the polymer difficult for the production of long-term delivery systems [20]. Hence, this adverse aspect requires the use of a crosslinking agent in forming non-soluble networks in microcapsules [24].

Additionally, the functional properties of gelatin can be divided into two groups. The first group is associated with gelling, for example, gel strength, gelling time, setting and melting temperatures, viscosity, thickening, texturizing, and water binding. The second group is based on the surface behavior of the gelatin, for instance, emulsion formation and stabilization (such as in marshmallow), protective colloid function, foam film formation, and adhesion/cohesion are in this group [23]. Gelatin (Figure 1.3) contains several glycine, proline and 4-hydroxyproline residues. A typical structure is Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro- [25].

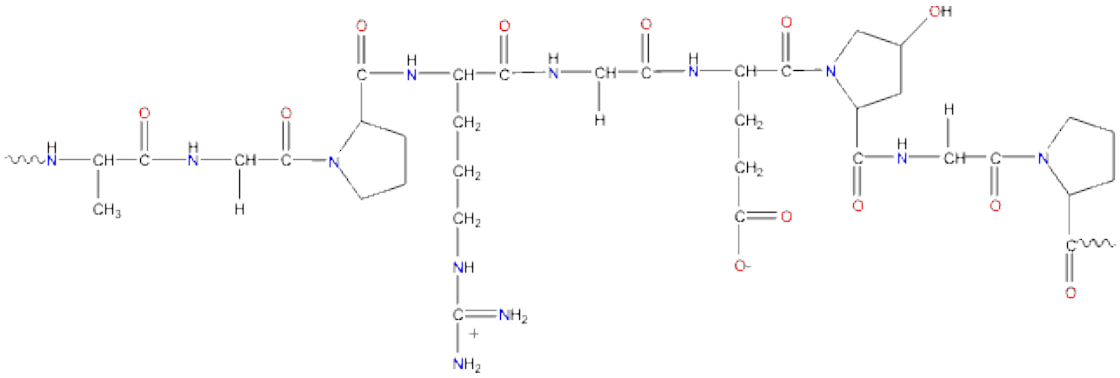


Figure 1.3 : A typical structure of Gelatin [25].

Gelatin, which is obtained by thermal denaturation or physical and chemical degradation of collagen, is commonly used in complex coacervation [1,26]. It is derived from animal skin, white connective tissue, and bones [23] and could be prepared by the hydrolytic degradation of collagen under either acidic or alkaline

conditions. Based on the processing conditions, there are two types of gelatin produced commercially: type A is normally prepared by (1–2 days) acid treatment of pigskin, and type B by alkaline treatment of bones or cattle hide with lime (calcium hydroxide) for 1–3 months [27]. Type A and type B gelatin are resulted by the partial cleavage of protein cross-links [23]. It is completely resorbable in vivo and its physicochemical properties can be suitably modulated; moreover, it is much cheaper and easier to obtain in concentrate solutions [26]. Since collagen is originated from animals, it must be seriously investigated before it can be used in humans because of its antigenicity even, it is essentially denatured collagen and has relatively low antigenicity [21].

1.4.2 Gum Arabic

Generally, proteins act as the main stabilizers and polysaccharides help to emulsion stability by their thickening and steric stabilizing behaviors [28]. Polysaccharides have important roles in many foods as a thickener, stabilizer, and gelling agents because of their hydrophilicity, highly-branched structure and high molecular weight [29]. In the food industry, gums are the commonly used polysaccharides. The term “gum” most often specifically represents a group of industrially useful polysaccharides or their derivatives that hydrate in hot or cold water to form viscous solutions or dispersions at low concentrations. Natural gums include seaweed extracts (e.g. alginates), plant exudates (e.g. arabic and tragacanth gums), gums from seed or root (e.g. potato starch), and gums obtained by microbial fermentation (e.g. gum xanthan) [25].

Gum Arabic (GA) or gum acacia, which is a gummy secretion from the stems or branches of Acacia species like Acacia senegal, Acacia seyal or Acacia polyacantha, is one of the widely used polysaccharide because of its natural origin, non-toxicity, biocompatibility and biodegradability [30,31]. It is highly souluble in water and has ability to create a strong protective film around the oil droplet, and it produces low-viscosity solutions at high solid concentrations [29,32,33]. Gum arabic is mostly used in the food industry for structural stabilization and encapsulation (i.e., wrapping of the interface), as well as control of texture, color, retention and protection of chemically reactive and volatile oils and flavors [30,33]. In addition, gum arabic, is mainly used in oral and topical pharmaceutical formulations as a suspending and

emulsifying agent [32] and more than one half of the world's supply is used in sweets to decelerate sugar crystallization and to thicken candies, jellies, glazes and chewing gums [31].

Gum Arabic is defined as a highly branched arabinogalactan polysaccharide, containing about 2% protein [34], and both protein and polysaccharide parts are fundamental to the functional features of this polysaccharide. It allows a macromolecular barrier against destabilizing mechanisms by increasing the viscosity of the aqueous phase and slowing flocculation and (re-) coalescence between dispersed droplets [29,35]. Briefly, it consists mainly of a mixture of arabinogalactan (AG) (80–90% of the total gum in weight), glycoprotein (GP) (2–4% of the total gum in weight), and arabinogalactan protein (AGP) (10–20% of the total gum in weight) fractions. Structurally, the carbohydrate part of gum arabic is highly branched molecule consisting of a (1→3) β-D-galactose core with extensive branching through 3- and 6-linked galactose, and 3-linked arabinose. Other carbohydrate contents include galactopyranose, arabinopyranose, arabinofuranose, rhamnopyranose, glucuropyranosyl uronic acid, and 4-O-methyl glucuropyranosyl uronic acid [34,36]. The carboxyl groups of uronic acid are deprotonated in its normal ionized form near neutral pH values. GA carries a net negative charge for pH value above 2.0 by its glucuronic acid residues. The AGP fraction has been considered to be responsible for the emulsifying properties of GA. Indeed, the protein component of the gum would embed in the oil phase while the carbohydrate component would extend into water [28,34].

1.5 Crosslinker Agents

The choice of conditions such as pH, temperature and wall material according to core material is crucial for encapsulation but not enough. In order to overcome the rapid release of active material, for example, crosslinkers are generally used. During the microencapsulation procedure, the coacervates formed usually have low mechanical strength due to the ionic nature of the interaction between the polymeric layers [10]. In other words, without crosslinking microcapsules swell in an aqueous environment, collapsed and then stuck together [24]. To solve this problem, microcapsules are usually crosslinked and hardened by the addition of a chemical agent (crosslinking agents) via the formation of intra and intermolecular bonds [10,21]. Crosslinking, not

only improves the thermal and the mechanical stability of the material [26], but also microcapsules' swelling degree appeared to be lower with an increasing the degree of crosslinking [24]. Depending on the desirable degradation properties, different cross-linking treatments, including chemical and physical methods can be applied [21]. Physical crosslinking methods commonly involve the use of ultraviolet, gamma irradiation [26], drying and heating treatmets [21]. In chemical cross-linking methods, cross-linkers are used to bond functional groups of in the molecules like hydroxyl, carboxyl or amino groups. Glutaraldehyde, formaldehyde, epoxy compounds [37], tannic acid, dimethyl suberimidate, carbodiimides and acyl azide are generally used as crosslinking agents [21].

1.5.1 Glutaraldehyde

Aldehydes, such as glutaraldehyde and formaldehyde, are generally used to create a more rigid wall structure for controlled release studies [10]. Glutaraldehyde (GTA) (Figure 1.4), which is a highly reactive dialdehydes reagent [38], is easily available, inexpensive and its aqueous solutions can effectively crosslink in a relatively short period [39]. Among the chemical crosslinking agents, glutaraldehyde is the most extensively used, because of its high efficiency for stabilization of collagenous materials [39] as a fixative and crosslinker in biological assays [40,41]. It involves the reaction of free amino groups of lysine or hydroxylysine amino acid residues of the collagenous materials with the aldehyde groups of GTA [26].

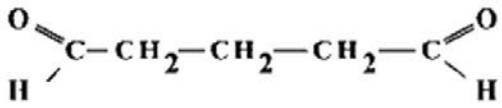


Figure 1.4 : Chemical structures of glutaraldehyde [40].

Furthermore, it is a high production volume chemical which has many medical and industrial uses [42]. Glutaraldehyde is mostly used as a general industrial antimicrobial in applications such as pulp and paper manufacture, water treatment, tanning, oil field, and other operations. It is used as an indirect food additive for several uses and in the medical field for cold sterilization of surgical instruments and endoscopes, X-ray film processing, and as a biological tissue fixative [38]. However, the toxicity of aldehydes cause to limitation of using it as a crosslinker for food and pharmaceutical applications [10]. This is the reason why the increasing demands for

a crosslinking agent able to form stable and biocompatible crosslinked products, without added cytotoxicity problems [26].

Monsan et al.[43] studied the mechanism of interaction of glutaraldehyde with protein and Navarro and Manson [43] studied the mechanism of interaction of glutaraldehyde with microorganisms; they concluded that as a result of glutaraldehyde reaction with a primary amine group, an imine bond is immediately formed [43]. Hence, the major reaction products of glutaraldehyde and amino compounds in aqueous solution at neutral pH are conjugated Schiff's bases [Figure 1.5(a)]. With glutaraldehyde polymerization, subsequent to fixation, a network crosslinking structure can be created intra- and inter- molecularly within, for example, collagen fibers [Figure 1.5(b)]. In polymerization, the aldehyde functional groups of two glutaraldehyde molecules may undergo an aldol condensation [Figure 1.5(b)] [40].

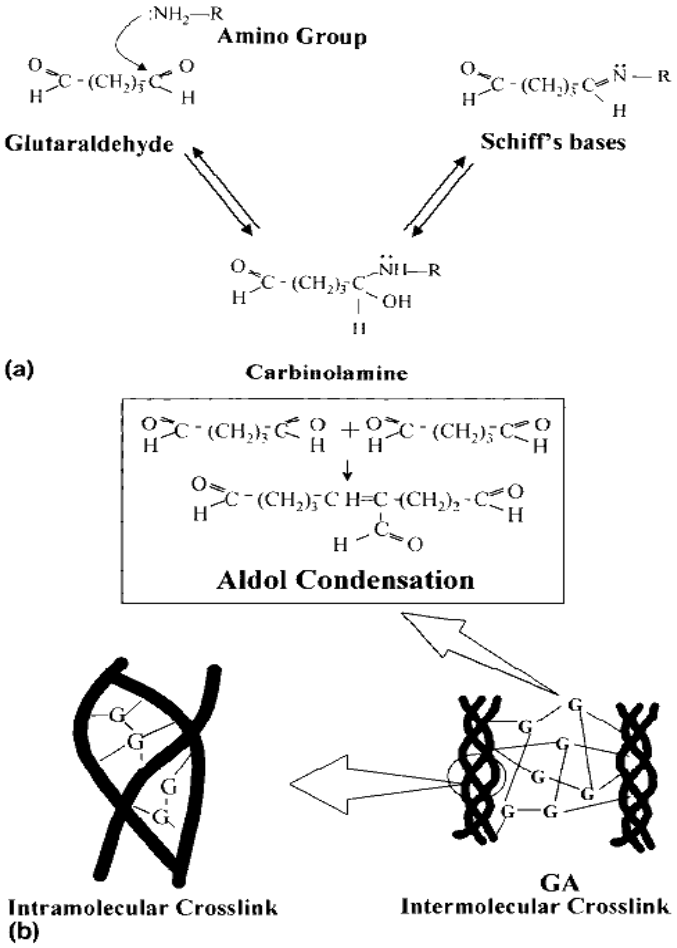


Figure 1.5 : (a) Presumable reaction mechanism of amino group with glutaraldehyde, (b) Schematic illustration of the presumable intramolecular and intermolecular crosslinking structures of the glutaraldehyde-fixed tissue [40].

1.5.2 Genipin

In earlier studies, synthetic bi-functional reagents like glutaraldehyde, formaldehyde and epoxy compounds are reported to be used [22] for microcapsule preparation to enhance membrane resistance and delivery features and controlled release [44]. However, the use of crosslinking agents such as formaldehyde and glutaraldehyde can lead to potential toxic side effects owing to residual crosslinkers [20] and also cause insufficient biocompatibility. This leads to an increasing requirement for a naturally derived reagents as an alternative [44] crosslinking reagent which are able to form stable and biocompatible crosslinked products with less cytotoxicity problems [22].

In order to overcome this problem, a naturally occurring, water-soluble bi-functional [45] crosslinking agent genipin seems to display promising characteristics [26], mainly low cytotoxicity [46] and biocompatibility [20]. Especially, studies showed that, genipin significantly less cytotoxic than glutaraldehyde, for instance, it may provide a less extent of cross-linking to form a semi interpenetrating polymeric network (semi-IPN) [47]. The cytotoxicity of genipin compared to glutaraldehyde in vitro using 3T3 fibroblasts with the MTT assay [44]. It had been showed that genipin was about 10,000 times less cytotoxic than glutaraldehyde. Furthermore, the colony forming assay demonstrated that the proliferative capacity of cells after subject to genipin was approximately 5000 times greater than cells subjected to glutaraldehyde. Additionally, the degradation rate of the genipin-crosslinked microcapsules was significantly slower than their glutaraldehyde crosslinked counterparts [24].

Genipin (Figure 1.6), which is an aglucone [44], can be obtained from its parent compound, iridoid glucoside [26], geniposide, which is found in the fruits of Gardenia jasminoides ELLIS [21,47,48]. It is a component of a traditional Chinese herbal medicine [48] and abundantly present in gardenia fruits [26]. Genipin and its related iridoid glucosides have been widely used as an antiphlogistic and cholagogue in herbal medicine [21,40]. Genipin has been reported to bind with biological tissues and biopolymers such as chitosan and gelatin, leading to covalent coupling [44]. Additionally, it is known that genipin can spontaneously react with amino acids or proteins like gelatin to make dark blue pigments. These dark blue pigments are currently used as a natural colorant in the food and fabric industries [44]. Furthermore, it was found that genipin can react with the free amino groups of

lysine, hydroxylysine, or arginine residues within collagen-based biomaterials [20,22].

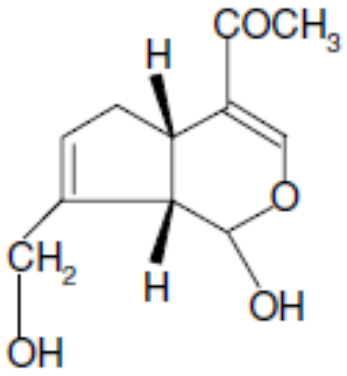


Figure 1.6 : Chemical structure of genipin [48].

In another study, chitosan matrices have been successfully cross-linked with genipin [45]. It was also found that the genipin-fixed tissue had a resistance against enzymatic degradation comparable to the glutaraldehyde-fixed tissue and can form stable crosslinked products [40]. Other applications of genipin involve the preparation of microcapsules [26], ideal for clinical usage [44] and the immobilization of enzymes [26]. Furthermore, gelatin-derived bio-adhensives display higher biocompatibility and less cytotoxicity when cross-linked with genipin than with other agents [21]. It was reported by Touyama et al. that only primary amines, rather than secondary or tertiary amines, can react with genipin [47]. The mechanism of the reaction of amino acids or proteins with genipin is still not well understood at present [21]. Touyama et al. studied the structures of the intermediates leading to a blue pigment produced from genipin and methylamine, the simplest primary amine [47].

Briefly, the mechanism proposed by Touyama's group for the formation of the genipin-methylamine monomer is through a nucleophilic attack by methylamine on the olefinic carbon at C-3 in genipin, followed by the opening of the dihydropyran ring and an attack by the secondary amino group on the resulting aldehyde group. The blue-pigment polymers are presumably formed through the oxygen radical-induced polymerization and dehydrogenation of several intermediary pigments (Figure 1.7) [21], similar results were also reported by Fujikawa et al. [47]. The results of these studies suggest that genipin may react with free amino groups and form a tertiary amine structure which is more stable than Schiff base Figure 1.7(a)

and may form intra- and inter-molecular crosslinks with cyclic structures within collagen fibrils in biological tissue Figure 1.7(b) [21].

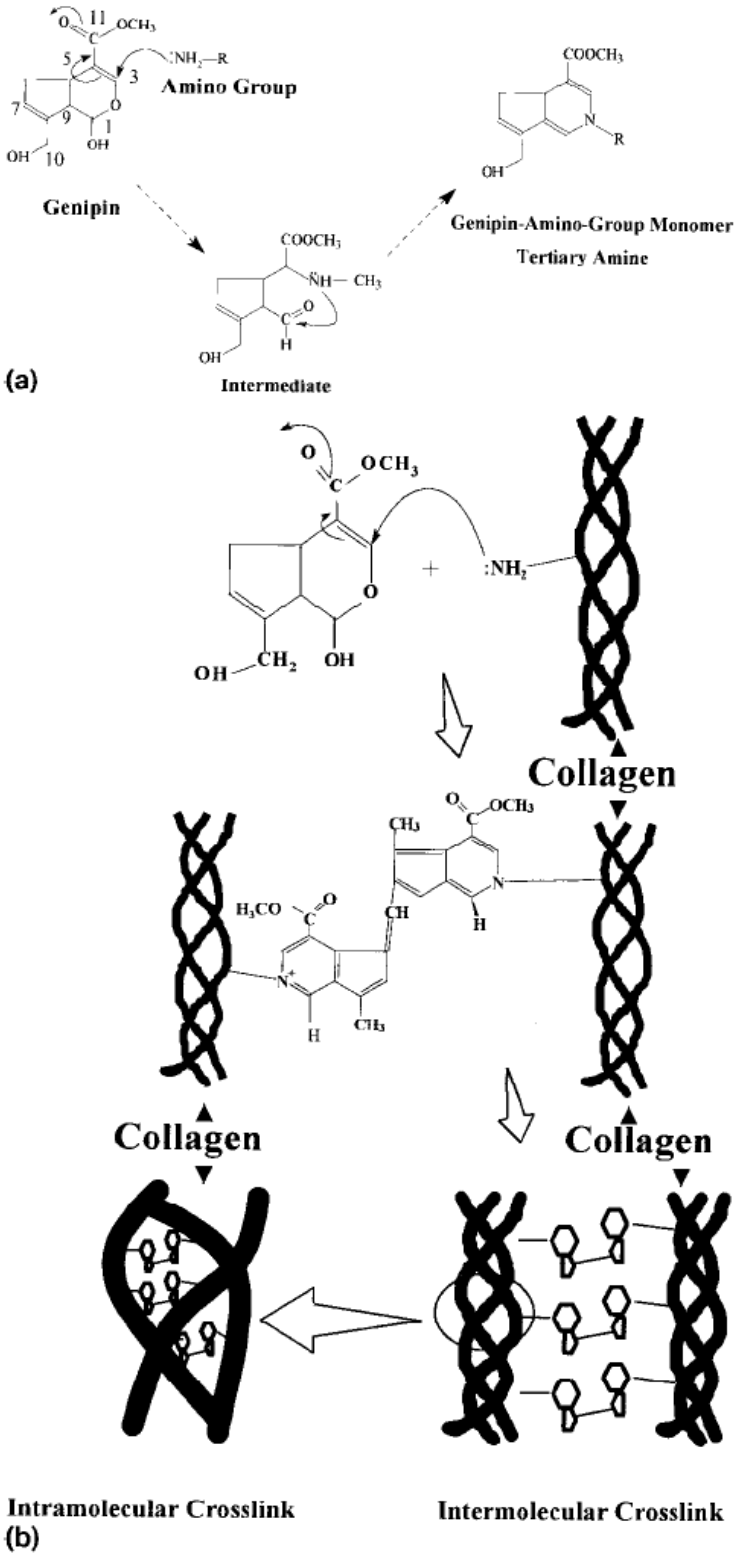


Figure 1.7 : (a) Presumable reaction mechanism of amino group with genipin. (b) Schematic illustration of the presumable intra- and inter- molecular crosslinking structures of the genipin-fixed tissue [40].

1.6 Flavors

Flavors or aromas are known to be the most important characteristics of food products. Materials used as aromas are usually consisted of many volatile and odorous organic species [49]. They play important roles in consumer satisfaction and influence the further consumption of foods. However, the unstability of flavors in foods make it difficult to preserve them for desired period of times so there is a growing interest to the control of stability of them with different approaches [13].

Flavor compounds are very heterogeneous group varying greatly in their properties. There are neutral compounds, acids, nitrogen, and sulfur compounds and compounds with high and low volatility, which influence the flavor of a food product [2]. Moreover, flavors form very complex systems to work with because there are many variables affecting their final properties. Some are water soluble and more stable in carbohydrates and some are more stable in lipid-based coating. There are many factors related to aroma affect the overall quality of the food, for example, physico-chemical properties, concentration and interactions of volatile aroma molecules with food components [1]. Food products including synthetic flavors are often unwanted, because the consumers suspect that these compounds are toxic or harmful to their health, yet unfortunately most available aroma compounds are produced with chemical synthesis or extraction [1]. Furthermore, most liquid food flavors are volatile and chemically unstable in the presence of air, light, moisture and usually thermally sensitive compounds, which require special treatment during food processing [49]. So, production and storage processes, packaging materials and ingredients in foods often cause modifications in overall flavor by reducing aroma compound intensity or manufacturing flavor components [1]. Hence, it is useful to microencapsulate volatile ingredients prior to use in foods or beverages to limit the degradation of aroma compounds during production, storage and/or transport could be critical in terms of stability and quality [13]. The orange oil was extracted from the peel of citrus at normal temperature, using the cold pressed oil technique. The chemical structure of orange oil is shown in Figure 1.8 [50]. The distillation temperature for orange oil is 175^0C. The main ingredient of orange oil was D-limonene which is ~ 90%. Orange oil can be used for many applications. It may be used as an agent or a source in surface cleaners, hand cleaners, furniture polish, soaps and shampoo production.

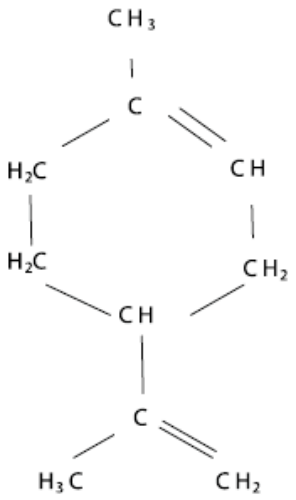


Figure 1.8 : Chemical structure of orange oil [50].

2. MATERIALS AND METHODS

2.1 Materials and Equipments

2.1.1 Chemicals

The chemicals and their suppliers are given in Appendix A.1.

2.1.2 Equipments

The equipments are given in Appendix A.2.

2.2 Methods

2.2.1 Preparation of microcapsules

Microcapsules were prepared by complex coacervation method using the following process, adepted by Leclerq et al [16]. Briefly, gelatin (3 g) and gum arabic (2 g) were dispersed in 112.5 mL of distilled water heated at 45 °C in a beaker and the solution was homogenized using an overhead stirrer rotating at 350 rpm. pH of the solution was adjusted to 4.5 with hydrochloric acid (10 % aqueous solution). Corn oil (as the unflavored liquid core material) was added into the solution after 30 min and the stirring rate increased to 600 rpm for 25 min for better emulsification, maintaining the temperature at 45°C. After emulsification, 100 mL of distilled water (35 °C) was added and the stirring rate was reduced to 300 rpm, and the system was slowly cooled down to 13 °C. Cooling procedure was performed in two steps. In the first step, the room temperature (ca. 26 °C) was reached in ca. 1.5 h by removing the heating source and in the second one, a water bath filled with iced water was used to cool the solution to 10-13 °C (ca.1 h).

2.2.2 Effect of wall materials (gelatin/gum arabic) ratio

Gelatin and gum arabic were mixed in four different ratio to obtain a total of 5 g wall material for each experiment (Table 2.1).

Table 2.1: The gelatin / gum arabic ratio used in the preperation of microcapsules.

Chemicals	**Ratio (w/w)**			
Gelatin/Gum Arabic	3:2	2:3	1:1	4:1

2.2.3 Effect of core material (corn oil)

In the encapsulation step, gelatin/ gum arabic ratio kept constant at the 3:2 and the core material amount was changed as 12 mL, 22 mL and 33 mL.

2.2.4 Effect of strirring rate

In the encapsulation step, gelatin/ gum arabic and core material ratio kept constant at 3:2 and 22mL, respectively and the emulsification step was carried out at different stirring rates as 400 rpm, 600 rpm, and 800 rpm.

2.2.5 Preparation of microcapsules with different cross-linkers

2.2.5.1 Glutaraldehyde as cross-linker

After the preparation of microcapsules, the cross-linking procedure was carried out, while maintaining the system at about 10-13 °C and stirring at 300 rpm. pH was adjusted to 9 with sodium hydroxide (5 % aqueous solution) and 452 µL of gluteraldehyde (50 %) was added on this solution as cross-linking agent. The cross-linking step was continued at ca. 2 h at 10-13 °C and the solution was then allowed to reach room temperature for the next 16 h.

2.2.5.2 Genipin as cross-linker

Prepared microcapsules were cross-linked with genipin with the following procedure. The mixing was stopped and it was waited for 1 h for the microcapsules to allow separation of oil encapsulated microcapsules from the empty ones. The microcapsules were then collected and washed with distilled water for several times. Genipin solutions (0.03 M, 0.01 M, 0.005 M, 0.002 M) were prepared and for 2 g of capsule, 4 mL genipin solution was added on this solution. Different duration times (ca. 20, 42, 66, 90 h) were tested for each concentration of genipin solution.

2.2.6 Conditions of freeze drying process

The freeze-drying technique, which is also called lyophilization, is one of the most useful processes for drying thermo sensitive substances that are unstable in aqueous solutions. In the freeze-drying process solvent is removed from a frozen solution by vacuum sublimation, maintaining the drying chamber pressure and temperature bellow the triple point of solvent. Freeze drying appears as one of the most suitable methods for dehydration of almost all heat-sensitive materials and aromas, due to the lower operating temperatures, slow drying rate and to the use of vacuum [51].

In this process, the crosslinked microcapsules were collected and washed with distilled water with the help of an extraction balloon. They were then transferred into petri dishes and cooled to -20 °C in a freezer. After 24 h, the frozen microcapsules were freeze-dried for 72 h, at chamber temperature of -39 °C and vacuum of 0.14 mbar.

2.2.7 Ninhydrin assay

The degree of cross-linking of gelatin/gum arabic microcapsules were determined by ninhydrin (NHN) assay. Ninhydrin reacts with free amino groups, e.g. NH_2-C-COOH, and a deep blue or purple color was produced as a result of the reaction. The assay determines the percentage of free amino groups remaining in the gelatin/ gum arabic microcapsules after cross-linking procedure. Ninhydrin solution was prepared by mixing solution A and B for 45 min and the content of these solutions were given on Table 2.2.

Table 2.2 : The solutions used for ninhydrin assay.

Solution A	**Solution B**
citric acid (1.05 g)	ninhydrin (1 g)
NaOH (10 mL, 1.0 M)	ethylene glycol monomethyl ether (25 mL)
$SnCl_2.2H_2O$ (0.04 g)	
In distilled water (final volume: 25 mL)	

For the assay, the microcapsules were lyophilized for 24 h and then weighed out. Lyophilized sample (1.5 mg) was put in 1 mL ninhydrin solution and the mixture was heated to 100°C in a water bath for 20 min. The solution was cooled down to room temperature, diluted with isopropanol (5 mL, 50 %), and then the optical absorbance of the solution at 570 nm was read on a spectrophotometer.

2.2.8 Encapsulation yield

In this step, the oil amount on the surface and inside the microcapsules was measured. The yield was calculated as the amount of encapsulated oil per total oil used in the experiment. Surface oil was analyzed with the following procedure: 3 g of dry microcapsules were weighed and washed with 10 mL hexane and the liquid phase was transferred in a volumetric flask. The hexane-oil mixture was evaporated by using rotary evaporator under the vacuum to separate hexane from oil.

In order to determine the encapsulated oil amount, the microcapsules were mechanically crushed by using a glass pestle in a mixture composed of chloroform (30 mL), methanol (15 mL) and distilled water (60 mL). The chloroform-oil mixture was collected with the help of separating funnel and chloroform was evaporated using rotary evaporator under the vacuum by 120 rpm stirring rate at temperature 60ºC.

2.2.9 Analysis of encapsulated flavors

Gas chromatography (GC), is a common type of chromatography used in analytical chemistry for separating and analyzing compounds based on their volatilities.

First the oil remained on the surface of microcapsules was analyzed. In order to remove the oil from the surface, 0.5 g of microcapsules was put into a 15 mL falcon tube and a total of 5 mL of heptane containing 1000 ppm of internal standard (dodecane) was added. The tube was then capped and vortexed at 2000 rpm for 2 min. A total of 3 mL of solvent was removed with a glass syringe mounted with a syringe filter (0.45 μm pores, nylon) to remove any remaining microcapsules. A total of 1 μL of the solvent was then injected into gas chromatograph with a flame ionization detector (FID) at operating conditions shown in Table 2.3.

Total flavor load of the microcapsules was analyzed using 1 g of capsules. The extraction was carried out in a 15 mL falcon tube by adding 3.5 mL of deionized

water containing 12.5 mg of protease. The mixture was heated to 60 °C for 5 min, and then placed on the shaker (450 rpm) at room temperature for 48h. The sample was allowed to rest for 1 h after shaking, and 3 mL of this mixture was transferred into a new 15 mL falcon tube. An equal volume of dodecane including 1000 ppm of internal standard (heptane) was added, which was then vortexed for 1 min. A total of 1 μL of the sample was then injected into gas chromatograph atoperating conditions shown on the Table 2.3. Oven temperature program used was represented on Table 2.4.

Table 2.3 : The GC operating conditions.

Injection Port (°C)	**Detector (°C)**	**Column Head Pressure (psi)**	**Split Ratio**
225 °C	250 °C	12 psi	1:50

Table 2.4 : Oven temperature program.

Temperature (°C)	**Duration (min)**
43°C	For 6 min
15°C min^{-1}/ 110°C	-
20°C min^{-1}/ 200°C	For 2 min.

Quantification was performed by dividing the peak area of the flavor compound by the internal standard and comparing this ratio to a pre-established calibration curve created under the same analytical conditions.

2.2.10 Visualization of microcapsules

2.2.10.1 Light microscopy

The structure and shape of microcapsules were observed by using a light microscope with a digital camera. The obtained images were used to determine the structure and wall thickness of microcapsules. All light microscope images were taken with 10X and 40X lens.

2.2.10.2 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is a rather new technique for structural analysis of biological and food material. CLSM allowed visualization of the polymeric particle wall composition using with different dyes. Furthermore, CLSM provides a method for three dimensional reconstruction and image analysis of the microparticles by imaging several sections throughout the object. It can be used to inspection of the encapsulated compounds and to detect special structural details of the particle wall composition [52].

Fluorescence labelling of the oil phase by nile red was done to be able to visulized the microcapsules using following protocol. The microcapsules were prepared using oil containing nile red with exicitation and emission wavelength of 553 and 633 nm, respectively. Since nile red shows a strong susceptibility to fluorescence bleaching, a minimum amount of 1 mg/ml of nile red, which is a relatively high concentration, was used. For this, 100µL dye was added to the core material.

Genipin also has a flourescense activity when cosslinked with microcapsules. Microcapsules which were crosslinked with genipin were visualized with CLSM, using an spectra from 380 to700 nm. All CLSM images were taken with a 40X lens.

3. RESULTS AND DISCUSSION

3.1 Effects of Different Wall Materials During Preparation of Microcapsules

In complex coacervation, the amounts of materials and their proportions are very important. Moreover, the size and the morphology of the microcapsules can be adjusted by selecting different core/shell weight ratio [53]. In this regard, different ratios of gelatin and gum arabic wall materials were used. During these experiments, core material (corn oil) was kept constant at 22 mL and total polymer amount at 5g in order to find differences between microcapsules. Increasing the amount of gelatin caused to the formation of a viscous mixture and a thick layer was formed on the surface during the crosslinking step at 13^0C. Furthermore, formed microcapsules were aggregated and due to gelation at room temperature, it became difficult to collect microcapsules from top of the system; there was no sufficient phase separation. Similarly, increasing the gum arabic ratio did not permit the formation of regular microcapsules (aggregates were formed instead). When gelatin and gum arabic amounts were the same, phase separation was observed but microcapsules remained suspended and eventually aggregated in the middle section of the solution. Table 3.1 shows encapsulation yields at different wall material ratios after drying.

Table 3.1 : Different gelatin/gum arabic ratio and encapsulation yields.

Sample			Encapsulation Yield (%)	Oil content (g/g microcapsule)
Gelatin (g)	Gum arabic(g)	Gelatin:gum arabic ratio(w/w)		
4	1	4:1	13.9 ± 2.0	0.2 ± 0.1
3	2	3:2	81.6 ± 5.0	0.4 ± 0.2
2.5	2.5	1:1	19.0 ± 1.4	0.3 ± 0.1
2	3	2:3	25.5 ± 19.0	0.3 ± 0.1

According to encapsulation yield, phase separation at the end of process and homogenization of microcapsules, the optimum wall material proportion was found

as 3:2 (gelatin:gum arabic; w/w). Leclercq et al. [16] has also found this ratio as their optimum condition and they concluded that process yield appears to be influenced more by the pH than by the ratio itself. Figure 3.1 also shows yield percentages based on ratio of gelatin/gum arabic. It could be seen that the yield percentage values of other proportions were quite lower than the optimum wall material proportion.

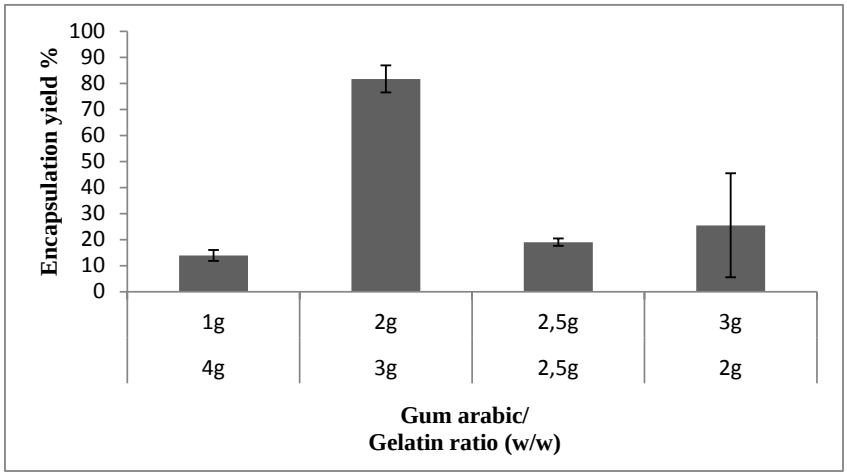


Figure 3.1 : Yield percentages of oil loaded microcapsules at different gelatin/gum arabic ratio.

3.2 Effects of Core Material (Corn Oil)

In order to determine the effect of core/wall material ratio to encapsulation yield, 12mL (10 g), 22mL (20 g), 33mL (30 g) corn oil were added to a constant amount of gelatin/gum arabic mixture (3:2 gelatin:gum arabic; w/w). It was observed that higher oil amount gave larger microcapsules while lower oil amount gave smaller microcapsules. In addition, higher core/wall material ratio may increase the thickness of wall membrane [54]. Table 3.2 shows the encapsulation yield at different core/wall material ratios.

Table 3.2 : Different amount of oil content and encapsulation yields.

			Encapsulation Yield (%)	Oil content (g/g microcapsule)
Gelatin/gum arabic (g)	Initial oil amount (g)	Gelatin/gum arabic:oil ratio (w/w)		
3:2	10	1:2	53.0 ± 15.5	0.7 ± 0.2
3:2	20	1:4	79.0 ± 1.4	0.4 ± 0.2
3:2	30	1:6	44.0 ± 11.0	0.5 ± 0.1

According to encapsulation yield, it was observed that using 20 g corn oil, as a core material, showed the most efficient wall material proportion in the encapsulation experiment (Figure 3.2). Dong et al. [14] indicated a significant size distribution differences in the multinuclear capsules formed depending on the core/wall ratio used. Based on Leclerq et al. [16] experiments a core/wall ratio of 4:1 resulted in an optimal shaped microcapsules.

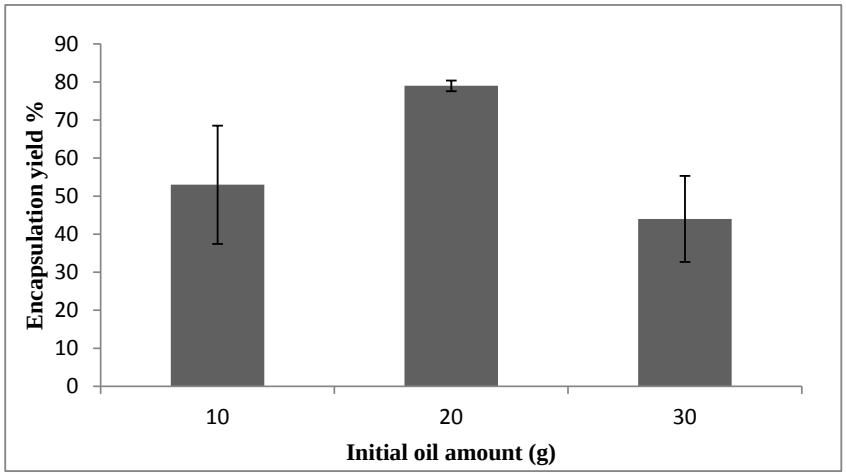


Figure 3.2 : Yield percentages of microcapsules loaded with different amount of corn oil.

At the end of experiments, no oil was observed in the emulsion medium during the preparation of microcapsules obtained by 10 g and 20 g oil, so the microcapsules were easily collected. A sticky texture was, however, observed with 30 g oil containing system because of the oil presence on the surface. Although wall thickness was not determined, information from the literature states that the wall thickness would decrease as a result of increasing core/wall material ratio and this, in turn, could effect the release rate. Leclerq et al. [55] represented that the release from coacervate microcapsules occurred by diffusion of aroma from the core through the wall, into the aqueous environment and they thought a thicker wall would slow the overall release but no statistical differences can be detected with microcapsules with different oil contents for both thinner and thicker wall.

3.3 Effects of Stirring Rate

It is worth mentioning that besides mixing ratio, there are some secondarily important parameters influencing the final microcapsules, e.g., stirring rate, temperature, hardening time, etc. These parameters should be optimized and may

vary from case to case [56]. In this study, different stirring rates from 450 rpm to 800 rpm were studied and encapsulation yield was measured. According to Salaün et al. [53], an increase of the stirring rate during the emulsion step will decrease the mean diameter of the obtained microcapsules. The reason is that under larger shear stress, their sizes will be smaller. Size distributions also strongly depended on the applied stirring rate [54]. In this study, the mean diameter was found to be ca. 250 µm at a stirring rate of 600 rpm. On the other hand, the increase of stirring rate to 800 rpm allowed to a sharp decrease in the mean particle size diameter (94 µm) while lower stirring rate led to the formation of particles with larger diameter (347 µm at 450 rpm).

Table 3.3 : The properties of oil loaded microcapsules (1/4, wall/core, w/w) at different stirring rates.

Stirring rate (rpm)	Particle diameter (µm)	Encapsulation Yield (%)	Oil content (g/g microcapsule)
450	347	35±3.5	0.6 ± 0.3
600	250	79±1.4	0.5 ± 0.1
800	94	20±7	0.5 ± 0.2

Briefly, Table 3.3 shows the effect of stirring speeds on microcapsule properties. The encapsulation yield varies according to the stirring rate applied during the emulsion step. In the literature, a low stirring rate and relatively high core amount was found to lead higher encapsulation yield [53]. In this study, however, an optimum stirring rate based on encapsulation efficiency was found as 600 rpm and microcapsules prepared using lower (450 rpm) and higher (800 rpm) speeds yielded lower values (Figure 3.3). Similar relationship between the stirring rate and encapsulation efficiency could be observed during the formation of drug-loaded coacervates and drug release [16,55]. In these studies, the stirring speed caused the differences in capsule structure (poly- , mononuclear) and particle size. During the emulsion step, the increase of the stirring rate decreases the diameter of the obtained microcapsules [53]. However, particle size was not found to be as important as wall thickness and capsule structure on influencing the rate of drug release [16].

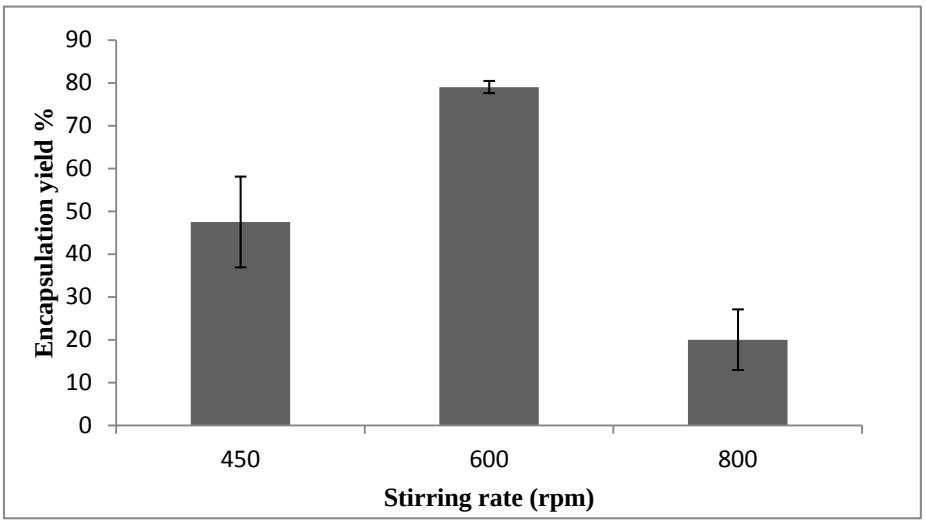


Figure 3.3 : Yield percentages of oil loaded microcapsules at different stirring rates.

3.4 Effects of Genipin

Genipin was selected as crosslinker because of its non-toxicity unlike glutaraldehyde. This compound is already being used for food dying agents in food industry. Due to these reasons, genipin could be a good alternative crosslinker for encapsulation of food derivatives. The reaction between microcapsules and genipin occurred after mixing the two solutions, and followed by the changes in the physical differences of the mixture. Microcapsules were first crosslinked using a non water soluble genipin. Dimethylsulfoxide (DMSO) was used to solve genipin and solutions with different concentrations (0.5mM-2mM) were prepared and used in microcapsule preperation. Reaction was carried out at two different temperatures, the room temperature and 30^0C. Blue color generation was expected after addition of genipin [46]. It was observed that the color of the genipin cross-linked gelatin/gum arabic microcapsules turned dark blue after 120h (5 days) at the room temperature when the genipin concentration was 2mM (Figure3.4). On the contrary, blue color was occurred within 72h (3 days) at 30^0C with the same genipin concentration (2mM). It could be said that reaction at the 30^0C was % 40 quicker than that occurred at the room temperature. Similarly, the speed and extent of the reaction were found to be dependent on reaction temperature by different groups [44]. This color change attributed to double bonds in the genipin cross-linked molecules [48]. However, water insoluble genipin require the usage of DMSO which is not desirable for food applications. For this reason, the experiments were also carried out by water soluble genipin at room temperature.

Figure 3.4 : Microcapsules crosslinked with genipin, at fixed time (72 h) but with different concentration (0mM, 0.5mM, 1mM, 2mM).

Briefly, blue color with high genipin concentration (30 mM), was obtained after an overnight incubation (20h) at room temperature while at low concentrations (2 mM), incubation time should be extended to 3 days and these findings were similar with the literature [48]. Blue color generation could be visualized after 9 h and as shown Figure 3.5, the color was intensified with increasing reaction time as observed in other studies [21].

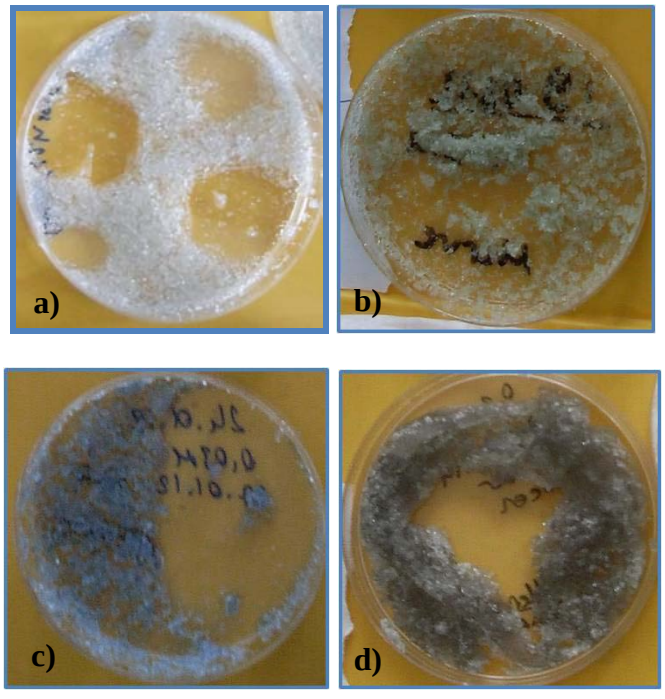


Figure 3.5 : The change in the intensity of color formation as a result of increasing reaction time from 12h to 90h at the same concentration (30mM), a) 12h b) 20h c) 42h d) 66h.

Table 3.4 summarizes details of the cross-linking reactions as a function of time and genipin concentration.

Table 3.4 : Effect of genipin concentration and reaction time on color formation.

Genipin concentration	Reaction time (h)				
	20	42	66	90	114
2mM	colorless	colorless	light yellow-green	gray-blue/ green-blue	dark gray-blue/ dark blue-green
5mM	colorless	colorless	light green	dark green-blue	—
10mM	light green	green-blue	dark blue-green	gelled blue	—
30mM	blue-green	dark blue	gelled dark blue	—	—

The color was darken with the increase of genipin concentration or cross-linking time at the room temperature and also gelation was seen as crosslinking time increases. However, capsules became partly gelled after a certain time and gelation degree gradually increases so the optimum conditions allowing fastest crosslinking without gelation should be determined [46]. Previous studies have demonstrated that an increase in the efficiency of the genipin reaction can also be reached by increasing the temperature and/or decreasing the pH [46].

3.5 Ninhydrin Assay

Ninhydrin assay was used to determine the amount of free amino groups remaining in microcapsules after crosslinking. Lyophilized sample was heated with a ninhydrin solution and the optical absorbance of the solution was recorded at 570 nm and the glycine at various known concentrations was used as standard. Figure 3.6 demonstrated that absorbance was increased with the increasing concentration of glycine.

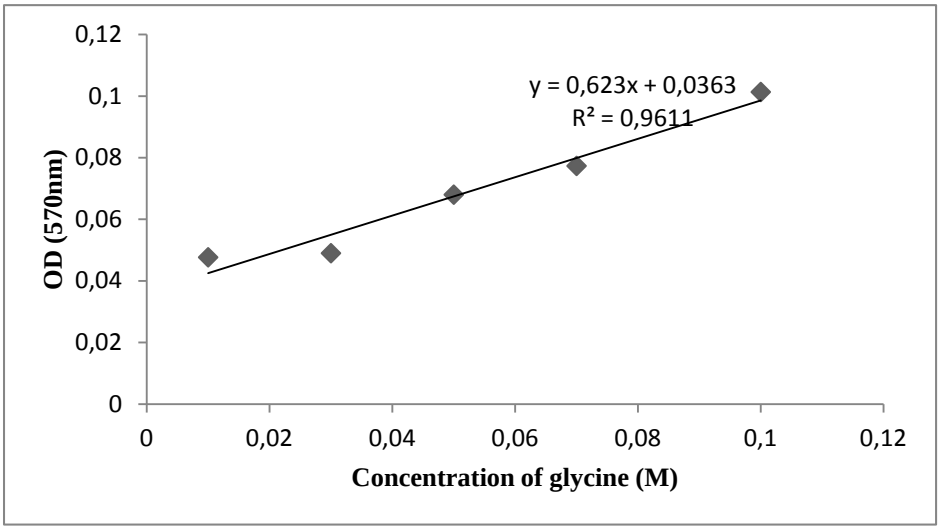


Figure 3.6 : Standard curve of glycine.

Figure 3.7 shows the amount of free amino groups obtained by ninhydrin assay for microcapsules crosslinked with genipin for 20 h. Although there is a difference in the color intensity, no obvious difference could be detected by ninhydrin assay at this conditions. It was thought that oil covered surface of microcapsules causing an inefficient reaction could be a reason for this unexpected result. Yao et al. [21] found that a higher concentration of genipin increases the degree of crosslinking. The reaction time was selected as 20h for our study to complete the reaction of

crosslinking. Also, Yao et al. [21] stated that at least one day was required to complete the cross-linking reaction.

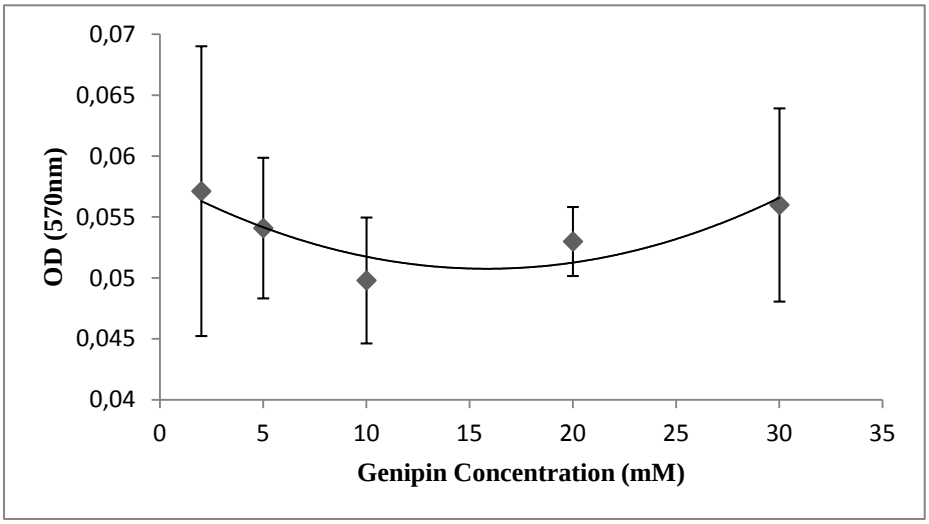


Figure 3.7 : Effects of genipin concentration on the croslinking.

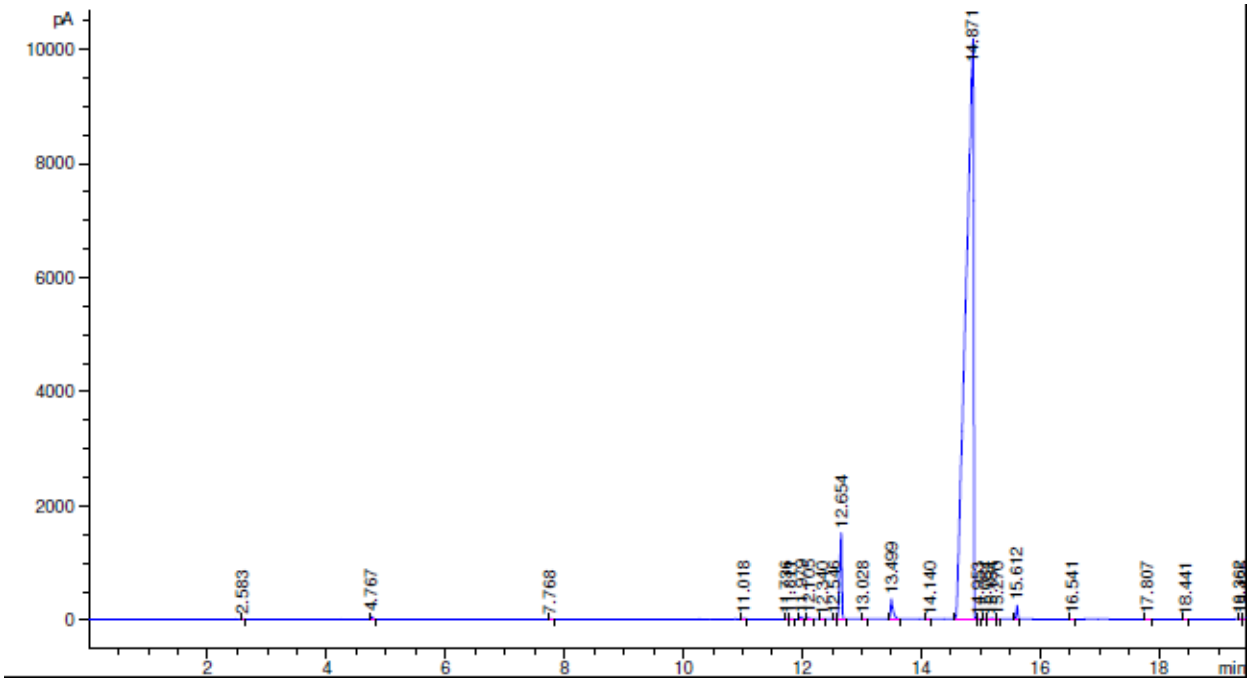
3.6 Encapsulation Yield of Genipin Crosslinked Microcapsules

After lyophilization of microcapsules, determination of encapsulation yield was done using 10mM and 30mM of genipin concentration. Encapsulation yields of microcapsules without a crosslinker and with a crosslinker (30mM genipin, 10mM genipin and 10,5 M glutaraldehyde) were found 78.8, 55.3, 57.8, 81.6% respectively. It was observed that increasing the genipin concentration 10mM to 30 mM did not significantly affect the encapsulation yield. Similar results were obtained from ninhydrin assay. Lower genipin concentrations did not give successful results due to the excessive aggregation of microcapsules.

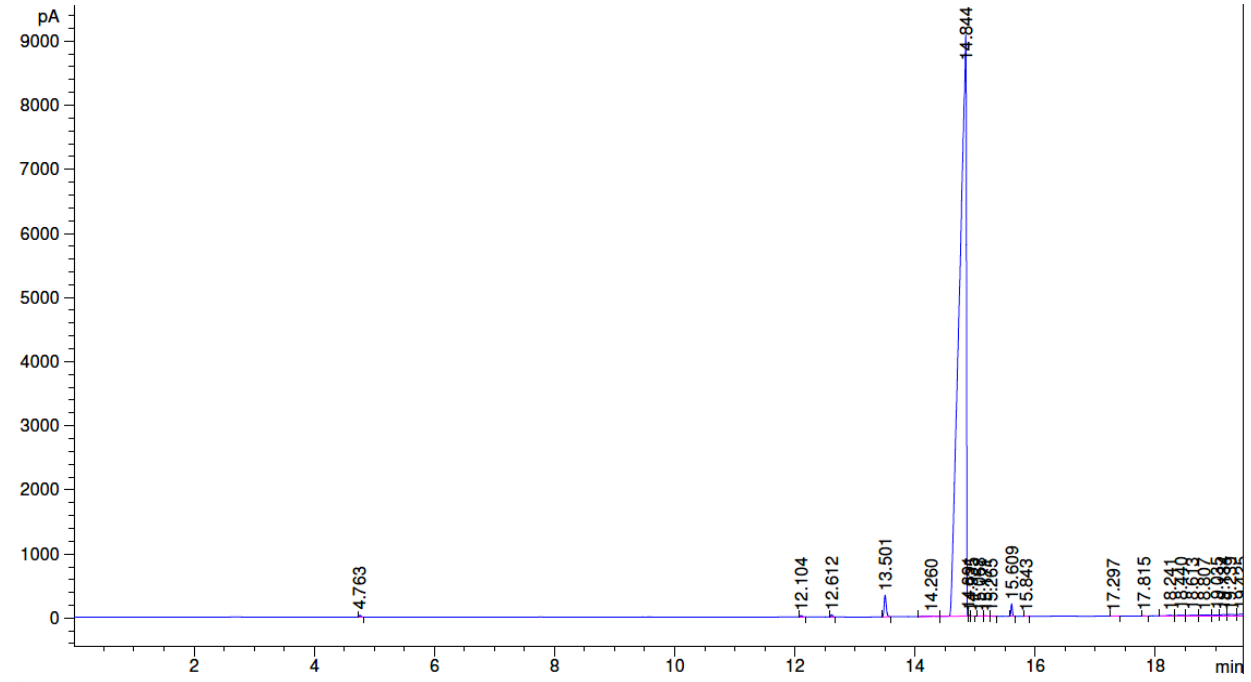
3.7 Analysis of Encapsulated Flavor (Orange Oil)

As opposed to corn oil, orange oil has high volatility, so affected by the environmental conditions such as temperature. Various amount of orange oil was used for encapsulation. However, at the end of the process, it was observed that considerably less microcapsules were formed when compared with corn oil loaded microcapsules and yield anlaysis were below 4.5 %. The corn oil and orange oil have different densities, 0.914-0.921g/ml, 0.842-0.846g/ml, respectively and this could affect the encapsulation procedure. Figure 3.8 shows gas chromatography results of unencapsulated with orange oil and encapsulated with orange oil. In Figure 3.8 (a)

there was observed significant peak at 12,654 for unencapsulated orange oil but in Figure 3.8 (b) there was not observed significant peak for encapsulated orange oil sample.



(a)



(b)

Figure 3.8 : Gas chromatography graph of unencapsulated with orange oil (a), and encapsulated with orange oil (b).

3.8 Visualization of Microcapsules

3.8.1 Light microscopy

The structure (mononuclear vs. aggregates or polynuclear) and size of microcapsules were investigated with optical and confocal laser scaning microscope. As shown in Figure 3.9 (a) and (b) microcapsules did not aggregate and they had a mononuclear structure. Moreover, it was observed that the samples had very similar external morpholiges and both spherical and rugby ball shapes could be seen. In Figure 3.9 (a) rugby ball shape microcapsules and in Figure 3.9 (b) smooth and slightly rough surfaces could be observed. Leclerq et al. explained that this different shapes are originated from fast stirring during the coacervation phase, inducing an alignment of the capsules with the water flow [16]. In addition, microcapsules could be damaged during drying process; smaller microcapsules were not much affected from the drying process but in larger ones, partial collapsing could be detected.

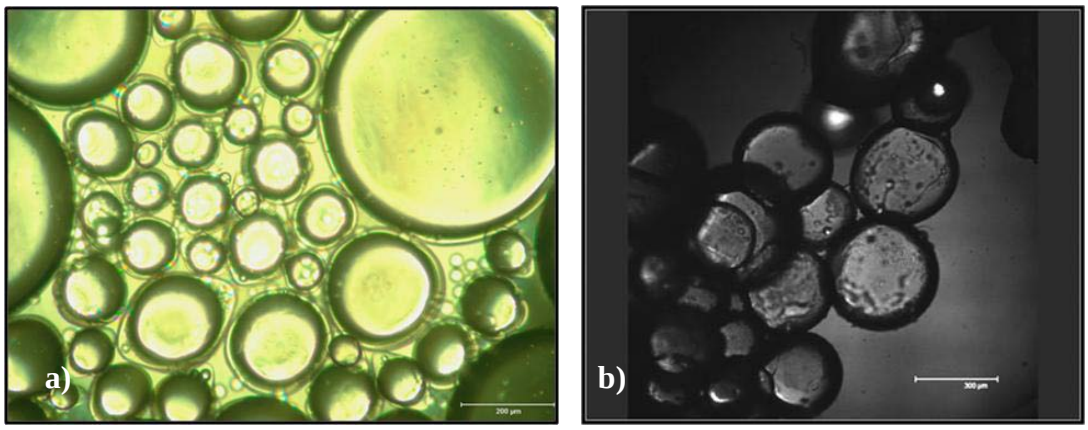


Figure 3.9 : Optical microscope images of microcapsules before (a) and after crosslinking (b) with glutaraldehyde. Scale bars 200µm (a), 300µm (b).

Many active agents like essential oils and flavors are instable compounds. They can suffer oxidation or volatilization or react with other formulation components. Those changes could explain the loss of flavor during encapsulation and a high amount of non-entrapped flavors [57]. The structure of coacervate microcapsules is significantly influenced by homogenization rate during emulsification process [14]. In Figure 3.10 it could be seen that there was half full microcapsules as expected, so instead of orange oil filled microcapsules, half filled microcapsules were obtained. The surface of the dried coacervate microcapsules was smooth and it presented a heterogeneously mixed populations as reported in [14]. It was observed that size

range differs between 100 µm to 250 µm and average size distribution was found as 148 µm which is lower than microcapsules encapsulated with corn oil. It is thought that volatility, temperature, density, emulsification step, etc. could be effective on these results.

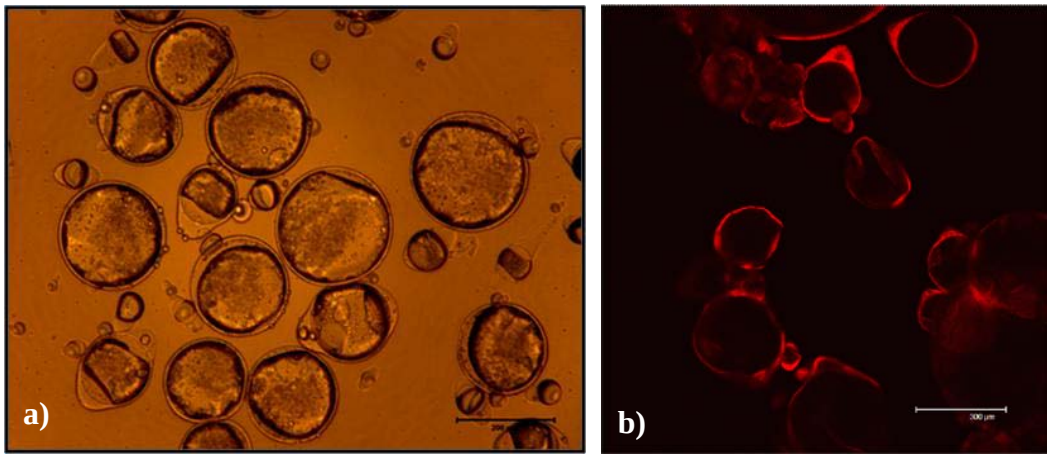


Figure 3.10 : Microcapsules optical microscope (a) and CLSM (b) images encapsulated with orange oil. Scale bars 200µm (a), 300µm (b).

Yuliani et al. [58] used extrusion instead of coacervation and mentioned that harsh condition during preperation can cause the loss of a large proportion of flavor volatiles due to thermal degradation, oxidation and reaction with food components. Once the inner core material volatized completely, the residual outer shell will be irregular which indicated that the inner oil has all exhausted through volatilization. Therefore, the oil release could be reasonably controlled by changing fabrication conditions [54].

3.8.2 Confocal laser scanning microscopy (CLSM)

Nile red is a lipid soluble, fluorescent dye [59]. Staining the oil phase with nile red allowed to separate between encapsulated oil and other droplet like structures [60]. In Figure 3.11, the transmission and fluorescence images of microcapsules stained by nile red were shown. Microcapsules were damaged while preparing the samples and it could be easily seen that nile red stained oil was leaking from the formed crevices. CLSM allows to identify all fluorescently labeled compounds at light in microscopical resolution unlike conventional light microscopy and nonfluorescent material cannot be detected without prior fluorescence labeling [60]. As mentioned in section 1.6.2, genipin can spontaneously react with amino acids or proteins and as a result of this reaction a blue colored and fluorescent product was produced [44]

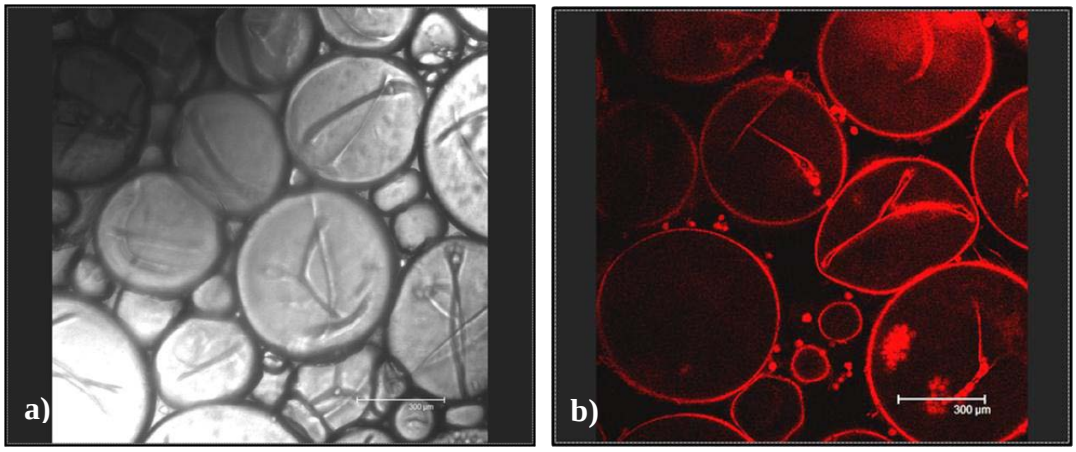


Figure 3.11 : Optical microscope (a) and CLSM images (b) of microcapsules encapsulated with nile red stained corn oil in the same batches. Scale bars 300µm.

and it was found that genipin concentration affected the fluorescence intensity of the product. In Figure 3.12 microcapsules were visualized as blue due to the genipin crosslinked microcapsules' fluorescent effect. Furthermore, it was observed that concentration of genipin affected the fluorescence intensity of microcapsule [44]. Mixture at the ratio of 1:2 (gelatin/gum arabic:genipin, w:w) gave higher fluorescense. Higher genipin concentration (30mM) fluoresced more than 20mM and 10mM, while at 5mM and 2mM of genipin concentration fluoresced very low.

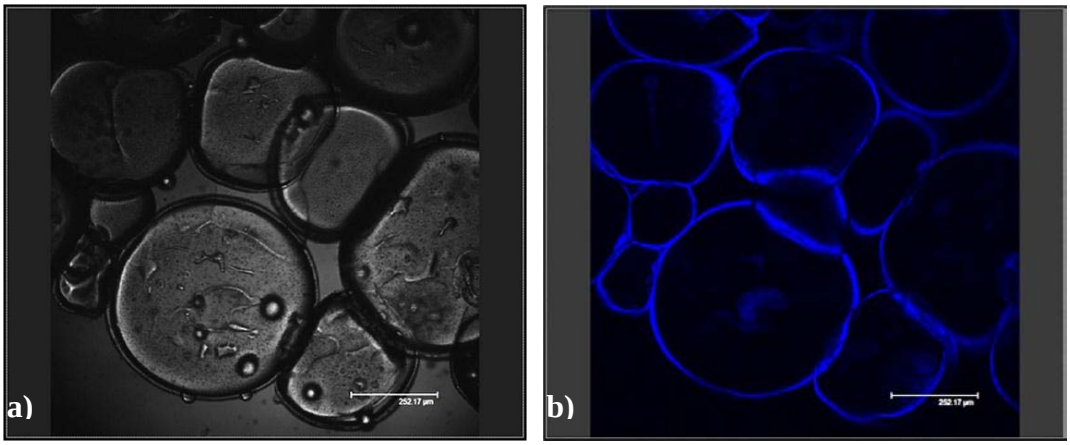


Figure 3.12 : Optical microscope (a) and CLSM images (b) of microcapsules crosslinked with genipin (30mM) in the same batches. Scale bars 252,17µm.

CSLM has been used to visualize heterogeneous distribution of the microcapsules such as different types of phase behaviour of mixtures of gelatin and polysaccharides [60,61]. Microcapsules encapsulated with nile red stained corn oil and crosslinked

with genipin was monitored via CLSM (Figure 3.13). The fluorescent images were taken separately for genipin crosslinked wall material and nile red stained core material and superimposed as seen in Figure 3.13.

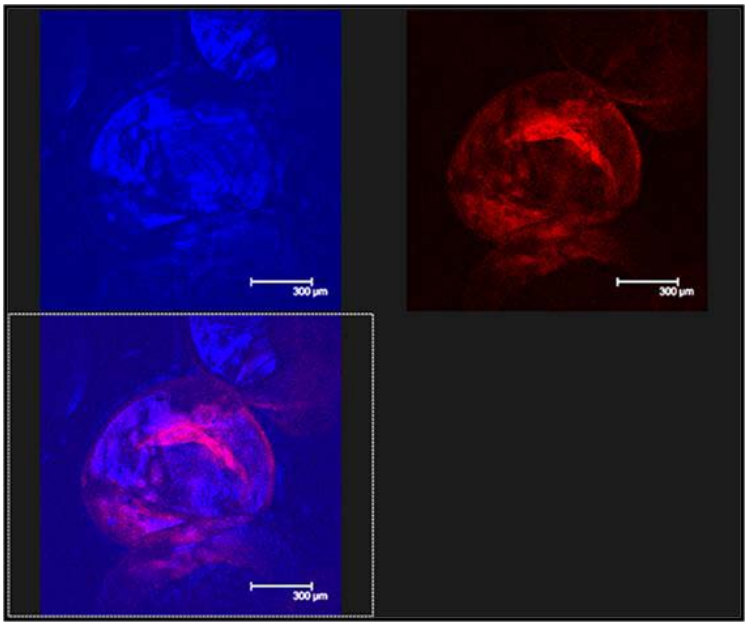


Figure 3.13 : CLSM images of microcapsules encapsulated with nile red stained corn oil and crosslinked with genipin (30mM). Scale bars 300µm.

4. CONCLUSION

Encapsulation is one of the effective methods which could be used in order to improve aroma quality and stability. The preservation of most liquid food flavorings, which are not only volatile and chemically unstable in the presence of air, light, moisture and high temperatures, but also are very expensive, is a complex problem. Encapsulation is an industrially important process for physically coating liquids, solids, or gases with thin, protective layer or wall of material to inhibit their loss by volatilization and to protect them against chemical deterioration.

In this study, the aim is to determine the potential of coacervation method and the usage of a natural crosslinker (i.e. genipin) in the efficient encapsulation of food flavors. Investigation of the effect of production conditions to microcapsule properties was also studied. As a model flavor, orange oil was encapsulated into different ratio of gum arabic/gelatin mixtures. Total amount of gelatin and gum arabic were kept constant but their ratio were changed. Highest encapsulation yield was found at 3:2 (gelatin/gum arabic, w/w). When gelatin or gum arabic amounts were higher than this optimum condition, newly formed microcapsules were aggregated and geleted. Apart from the ratio of wall materials, core/wall material ratio and stirring rates during emulsification were studied and it was observed that according to stirring speed, microcapsule size was changed such that increasing the stirring speed decreases the size of microcapsules. Prepared microcapsules were crosslinked by a natural agent, namely genipin, that is non-toxic unlike glutaraldehyde. Microcapsules were crosslinked with different concentrations of genipin solutions (2-30 mM in dH_20) and dried by lyophilization. Preparation conditions and different crosslinking agent concentrations were studied for their effect on coacervation properties. Crosslinking could be visualized by the blue color generation after genipin addition. Beginning of blue color generation was visualized nearly 8h later at 30mM genipin solution but at least an overnight incubation is needed to obtain an efficient crosslinking degree at room temperature. A longer

incubation period (3 days) was needed at low concentrations (2 mM). However, no significant differences was observed among different concentration of genipin (by ninhydrin assay) and also at the encapsulation yield results showed that microcapsules crosslinked with glutaraldehyde gave higher encapsulation yield than the ones crosslinked with genipin. Encapsulation yield for corn oil was determined to be 81.6 % for glutaraldehyde and 57.8 % for genipin (10mM) crosslinked microcapsules at optimum conditions. Microcapsules loaded with orange oil using genipin as a crosslinker gave very low yield (ca.4.5 %) and even in some experiments formation of microcapsule was not observed. It was also observed that environment conditions, especially temperature, could affect encapsulation process at each step of the procedure. Moreover, microcapsules were visualized in fluorescence microscopy using nile red as lipid staining dye. Size distribution for optimum conditions were found to be 250-350 μm. In conclusion, genipin was proved to be a suitable crosslinker for the encapsulation of food ingredients but the encapsulation procedure should be optimized to increase the yield for volatile compounds like orange oil. For future studies, different wall materials could be tried and crosslinking reaction with genipin could be performed at different pH values.

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APPENDICES

APPENDIX A.1 : Chemicals
APPENDIX A.2 : Laboratory Equipments

APPENDIX A.1

Chemicals	**Supplier**
Genipin	Wako Chemicals
Gelatin	Fluka
Gum Arabic	J.T. Baker
Glutaraldehyde (50%)	Sigma - Aldrich
Nile Red	Sigma - Aldrich
DMSO	Merck
Hexane	Sigma - Aldrich
Methanol	Sigma - Aldrich
Chloroform	Merck
NaOH	Riedel - de Haën
Glycine	Merck
Hydrochloric acid	Merck
Citric acid	Riedel - de Haen
Ethanol	Sigma - Aldrich
Tween 80	Merck
Dodecane	Sigma - Aldrich
Heptane	Sigma – Aldrich

APPENDIX A.2

Laboratory Equipments	Supplier
Deep freezers and refrigerators	Arçelik, 1061 M Refrigerator
Ice machine	Scotsman, AF 10
Magnetic stirrer	Cole Parmer, Stable Tempreature
Overhead stirrer	Ika Labortechnik, RW20.n
Micropipettes	Eppendorf AG, 1000µl, 100µl,10 µl, ,2500µl, 5000µl,
Precision weigher	Precisa, XB220A
Microplate spectrophotometer	Biorad, Benchmark Plus
pH-meter	InoLab, 720
Pure water systems	Elga Labwater, USF Elga UHQ-PS-MK3
Shaker	Heidolph, Duomax 1030
Vortex apparatus	Heidolph, Reax Top
Water bath	Huber, Compatible Control CC1
Rotary evaparator	Heildoph, Laborota 4000
Freeze dryer	Christ, Alpha 1-2 LD Plus
Gas chromatography	Agilent Technologies, 6890N
Confocal laser scaning microscope	Leica
Optical microscope	Olympus

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