PRELIMINARY ASSAY FOR EXTRACTION OF SAPONINS FROM STYRAX OFFICINALIS AND CHARACTERIZATION OF EMULSIFYING PROPERTIES OF THEIR EXTRACT

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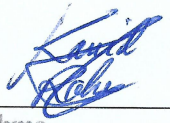

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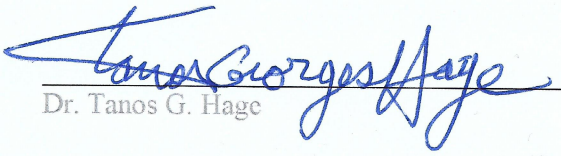

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ABSTRACT

An extraction protocol for saponins of *Styrax Officinalis* plant was adopted, modified and successfully applied. The extract obtained was tested by stabilizing Oil-in-Water emulsions. Ethanol extraction and successful one-step partial purification using AB-8 adsorption column chromatography yielded positive colorimetric (Vanillin – Sulfuric Acid) TLC results. The 3D structure of the four *Styrax Officinalis* saponins reported in the litterature were rendered for the first time, and their partitioning properties exclusively acquired using Marvin Sketch chemical drawing software. 10% w/w Sunflower Oil emulsions were prepared using our Styrax saponins extract and their properties compared to similar emulsions stabilized by analytical grade Acacia Gum. The saponins extract outperformed the Acacia Gum by making much smaller (180 ~ 900 nm compared to 5 ~ 1 µm for Acacia) and lower charged particles (ζ-potential ~ - 90mV) as measured by Dynamic Light Scattering. However, Styrax emulsion particles showed bimodal distribution and broader particle size DLS peaks (PDI ~ 0.4, compared to PDI ~ 0.1 for Acacia Gum). These results can be linked to the fact that the Styrax extract contains four different saponins as reported in the literature, each with its own emulsification properties. An attempt at assessing the antioxidant effect of the saponin extract of styrax and acacia gum on oil particles was made using the Rancimat method (accelerated ageing test). The preliminary results obtained showed that the Styrax extract would have good encapsulating effect on

the droplets yielding by longer induction times. However the induction times obtained had no direct correlation with emulsifier concentration. The oxidative stability experiments of *Styrax Officinalis* saponins are worth further investigations.

INTRODUTION

An increasing trend in the food, pharmaceutical, and cosmetic industry is the utilization of natural plant extracts or plant-derived compounds, as an alternative to the application of chemical or synthetic substances [1, 2]. This trend has been supported by the nontoxic nature of many chemicals in plants, their positive healthy attributes, consumer perception and acceptance of their use [3, 4]. Among different compounds derived from plants, saponins deserve a special mention. Saponins are amphiphilic glycoside compounds that derive their name from their soap-like properties. They are categorized into two groups according to the nature of their sapogenin moiety that can be either a steroid (C27) or a triterpenoid (C30) [1]. The carbohydrate part of the molecule consist either of hexoses, pentoses, or uronic acids [1]. Plant-derived triterpenoid saponins have been used in numerous industrial and commercial applications such as sources of raw materials for the production of steroid hormones in the pharmaceutical industry, food additives, photographic emulsions, fire extinguishers and denatured alcohol [2]. These applications take advantage of saponins' generally nonionic surfactant properties [5, 6]. The medicinal and health promoting properties of plants are generally correlated to the phytochemicals present in their secondary metabolite pool and the phytochemical studies may substantiate the ancient ethno-medicinal applications by giving a rationale from the chemical standpoint.

Styrax Officinalis is a deciduous large shrub that grows in southern Europe and the eastern Mediterranean region of Cyprus, Jordan, Lebanon, Syria, and Turkey [7]. It is an ichtiotoxic (toxic to fish) plant and its fruit extract has been historically used

in river fishing. It is also known for its antiseptic and expectorant resin obtained from its stems [8] and was used by the Romans, Egyptians, Phoenicians, and Ionians as incense and in therapeutics [9]. Earlier studies on the pericarps of *Styrax Officinalis* report the isolation of a triterpene sapogenin and four triterpenoid saponins [9, 10]. Other rarely occurring compounds were also identified such as benzoyl esters, anhydro-hexitols, and other potent chemicals that find diverse applications in the manufacturing industry [11]. A recent investigation also showed that the ichtiotoxic effect was related to the saponins fraction of *Styrax Officinalis* fruit pericarp [3]. This study also discovered a strong molluscicidal effect of *Styrax Officinalis* saponins on the terrestrial gastropods *gastropoda*. This explained the saponins' powerful disintegrating effects of on the soft membranes of the fish gills and snails. Moreover, S. Officinalis L. may be a useful source of enantiopure 1,5-anhydro-D mannitol which has several medicinal potentialities [4] and is a versatile building block in organic synthesis. It is specifically useful for bulk and fine chemical preparation of biologically active molecules, in particular for what concern the "Green" approaches. The amphiphilic nature of *Styrax Officinalis* saponins are believed to be the reason of their potent bioactivity as well as many attributes and applications that are yet to be discovered. Conducted research was limited to the mentioned information above without further quantification or investigation. As a continued work, this study aims at applying an optimized framework for the quantitative extraction and emulsification characterization of saponins from *Styrax Officinalis* fruit pericarp, in an attempt to valorize the promising aspects for these secondary metabolites. Also, the 4 Styrax Officinalis triterpenoid saponins reported in the litterature [5] were analyzed using Marvin

Sketch chemical drawing software and their physiochemical properties discussed in relation to their chemical structures.

CHAPTER 1 : LITERATURE REVIEW

1.1 OVERVIEW OF *STYRAX OFFICINALIS*

Styrax Officinalis L. is a member of the Styracaceae family [6]. It is a shrub found in Central America and the Mediterranean region. It has been traditionally used as incense, expectorant and condiment [6]. Its fruit is round shaped, yellow-cream to light green in color, covered with a velvety film. The fruit varies in size from 1 to 3 cm depending on the growing conditions and reach maturity late in summer. The fruit was also used by fishermen to stun fish in rivers and collect them from the surface of basin water. This has triggered interest in investigating Styrax's chemical profile that might be root of some specific bioactivity [3, 7]. Previous works have successfully isolated diverse compounds from the fruits including a sapogenin [8], four triterpenoids [5], benzofurans and several natural metabolites with chemotaxonomic relevance [7]. No previous studies on the total saponin content of the fruit of *Styrax Officinalis* has been recorded.

1.2 STRUCTURAL RELEVANCE OF SAPONINS

Secondary metabolites can be defined as a heterogeneous group of plant metabolites that are not essential for vegetative growth. They are considered differentiation compounds conferring adaptive roles such as: defense, signaling, symbiosis, metal

transport, competition, and so on [9]. Saponins are a class of naturally occurring substances with a rigid polycyclic skeleton of at least four hydrocarbon rings to which sugars in groups of one or two (up to ten) are attached. The name ‘saponin’ is derived from the Latin word ‘sapo’, meaning soap, as a soapy lather forms when plants containing saponins are agitated in water. They are amphiphilic compounds due to the presence of a lipid-soluble aglycone (Sapogenin) and hydrophilic water-soluble sugar chain. This provides efficient foaming properties (for gas-liquid phases dispersion), emulgator effects (for liquid-liquid dispersion) and dispersing abilities (for solid-liquid dispersion). Traditionally, they are subdivided into 2 categories:

Triterpenoid saponins which have a C30 sapogenin and Steroidal saponins that have a C17 tetracyclic sapogenin. They are further classified into mono-, bi- or tri-desmosidic depending on the number of hydrophilic sugar chains binded to the sapogenin (one, two or three respectively).

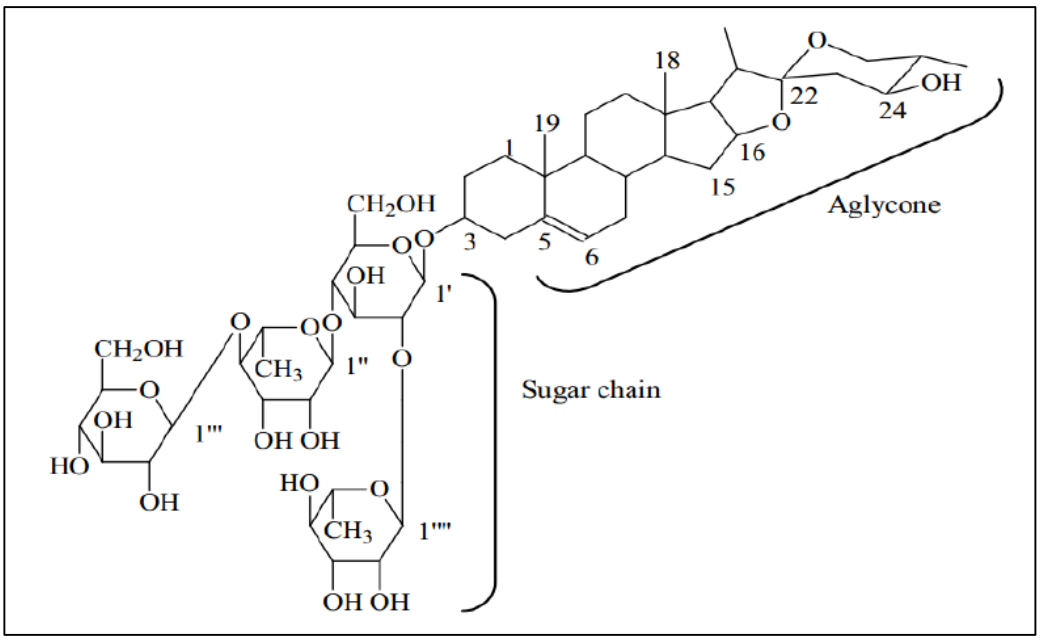


Figure 1: Monodesmosidic Triterpenoid Saponin [10]

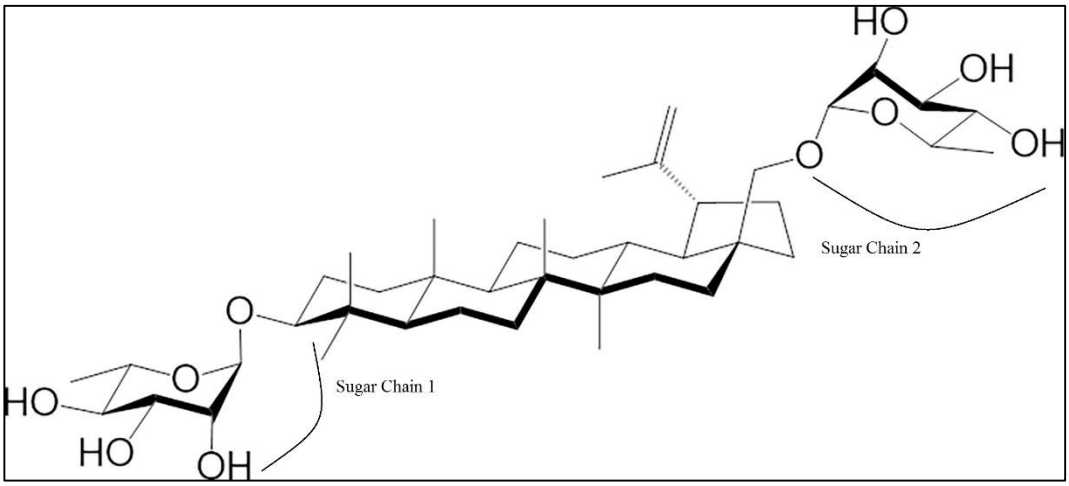


Figure 2: Bidesmosidic Saponin [11]

1.2.1 INDUSTRIAL USES OF SAPONINS

Saponins are among the most valuable plant secondary metabolites for industrial or biological applications. They possess sufficient chemical or structural complexity so that artificial synthesis is difficult or not currently possible [12]. Saponins have traditionally been used as natural detergents and are considered as a wide range of versatile molecules because of the multiple functional groups in their structure. They are also used as stabilizers in shampoos, conditioners, and in skin anti-aging products [13]. *Quillaja Saponaria*, native to China and several South American countries, was used as shampoo for hundreds of years, with extracts containing over 100 triterpenoid saponins. More recently, saponins are currently used as foam and emulsion stabilizers in beer and soft drinks, and as solubilizing agents for food additives. In the United States, *Quillaja* and *Yucca* saponin mixtures, among others, are classified as "food grade saponins" as approved by the US Food and Drug Administration (FDA). They are also Generally Recognized As Safe (GRAS) by the Flavor and Extract Manufacturers' Association of the United States (FEMA) with FEMA number 297 [2]. According to the Codex Alimentarius Commission,

the above mentioned saponins are used as a foaming agent in "water-based flavored drinks", including sport or electrolyte drinks and particulate drinks (GSFA category 14.1.4, 500 mg/kg maximum use level) [14]. Saponins are also know by their toxicity to harmful insects (anti-feeding, disturbance of the moult, growth regulation, mortality...) and are developed as potential biopesticides [15]. Steroidal saponins from *Dioscorea Mexicana* are essentially used in the pharmaceutical industry to synthesize progesterone since 1951 [16]. *Yucca Arborescens*, has been added to food as a 'shelf life extender' in the Japanese market. Yucca saponins are also used in confectionery/food industries for improving both product quality and shelf stability [1].

1.2.2 BIOACTIVITY OF SAPONINS

Saponins, in general, have been reported to have a wide variety of biological activities [1]. Pentacyclic Triterpenoids showed anti-cancer activity on prostate cancer tumors and various other tumor cells [17, 18]. Saponins from asparagus were proven to have cholesterolemic activity as tested in both animal and human trials [2, 19]. Ichthyotoxic, molluscicidal and insecticidal effect of saponins is well proven on freshwater fish, snails and insects [3, 15, 20]. It was suggested that 'lipid-A' (gram-negative specific lipid membrane)-saponin complexes could promote antibiotic (colistin, ampicillin) or disinfectant action toward inherently resistant microbial cells [21]. Potent immunostimulatory adjuvant, Quil-A®, is already being marketed as *Quillaja Saponaria* saponin-based vaccine by British firm Croda International. It is proven to induce strong cytotoxic CD8+ lymphocyte responses as well as cell- and antibody-mediated immune responses to a broad range of viral, bacterial, parasitic and tumor antigens[22-24]. Few saponins also showed hemolytic

activity that could restrain their use in some therapeutics [25]. This particular activity is shown to depend on the number of sugar units in the chains, with monodesmosidic saponins being more active [26]. One of the suggested mode of action was that cell membrane lipids would rearrange once the saponins would bind on the interface, forming sterol-saponin complexes. This will cause the formation of pores in the membrane and lysis of the cell [27, 28]. In food industry advancement, commercially available saponin extract successfully inactivated Gram-positive bacteria cells *Alicyclobacillus acidoterrestris* which is responsible for juice spoilage [29].

1.2.3 SAPONINS AS EMULSIFIERS

Saponins surface activity, due to their amphiphilic nature, is a key property for its application as an emulsifier. Saponins exhibit some very unusual surface properties, such as an extremely high surface modulus and shear viscoelasticity. These properties have an important impact on the mechanisms associated with foam and emulsion stabilization by these molecules. This was recently confirmed by Penfold et al. [13]. Penfold studied the adsorption layers of triterpenoid saponins at the water/air interface. It was concluded that triterpenoid saponin form dense steric packing (via strong intermolecular hydrogen bonds) at the interface giving rise to the observed high surface elasticity. However different binding mechanism were observed. Escin saponins (standardized analytical triterpenoid saponins mixture) showed a distinct ‘bend’ at the hydrophobic part of the molecule which resulted in the aglycone protruding into the water fraction. Tea saponins on the other hand binded perpendicularly to the air/water interface. Other works assessed detergency, foaming stability, wettability of triterpenoid saponins [30, 31]. These has further

proved the saponins' potency for commercial and industrial uses as emulsifiers and foaming agents.

1.2.4 *STYRAX OFFICINALIS* SAPONINS

Limited literature is available on *S. Officinalis.* Yayla et al. [5] isolated and determined the structure of four monodesmosidic triterpenoid saponins from the fruits of *Styrax Officinalis*. Three chemical screenings of *S. Officinalis* fruits [7, 32, 33] were later done and showed that seed contained steroidal saponins [8, 32] while pericarps contained triterpenoid saponins [5]. The four isolated saponins have a similar pentacyclic 'oleanane' aglycone and a 4-unit sugar chain. The difference lies in their functional groups on the hydrophobic part. Further detail on their specific individual properties will be detailed in section 3.1. However, the study made by Yayla et al. [5] focused on structure elucidation rather than characterizing the saponins. Anil [8] previously isolated a functionalized pentacyclic aglycone 'Barringtogenol-C' which is a known analgesic and anti-inflammatory [34]. Figure 4, 5, 6 and 7 are exclusive to this document and were rendered based on Yayla's work, but using chemical drawing tool Marvin Sketch version 19.19 (see Section 3.1).

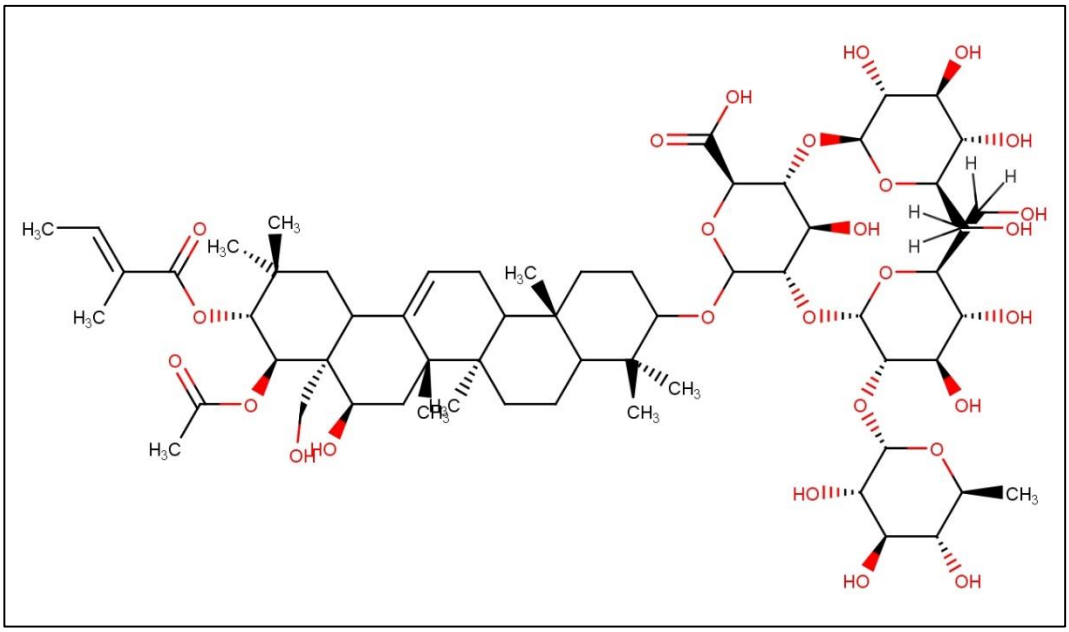


Figure 3: Saponin A

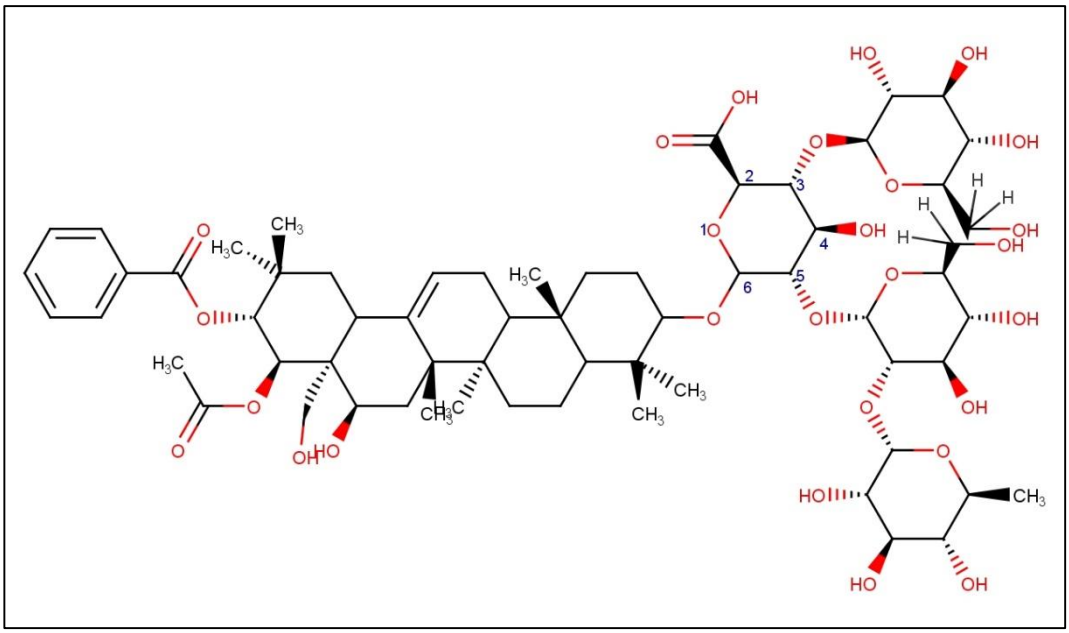


Figure 4: Saponin B

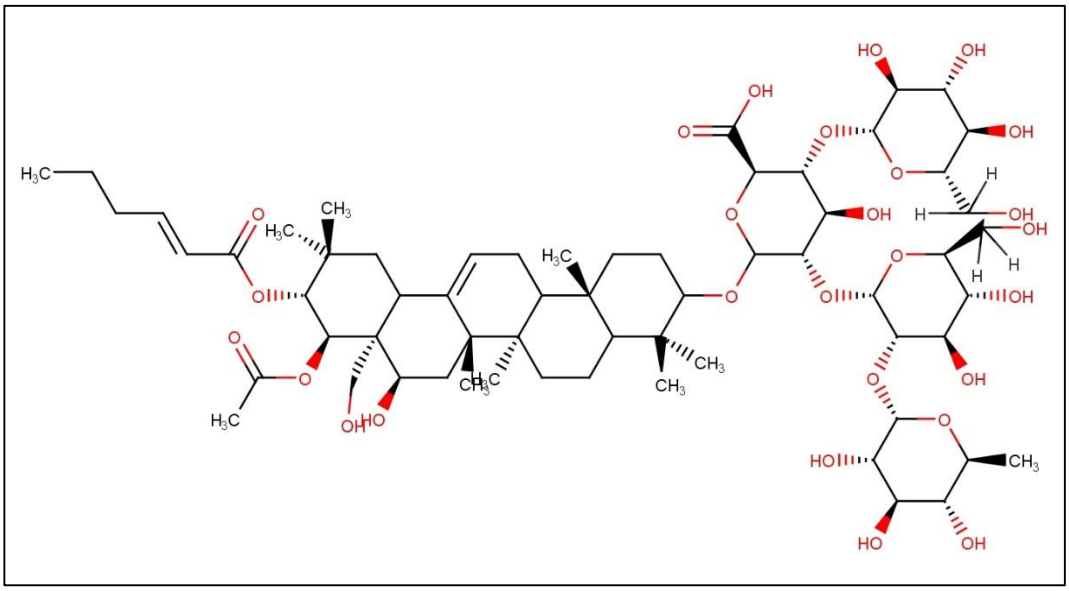


Figure 5: Saponin C

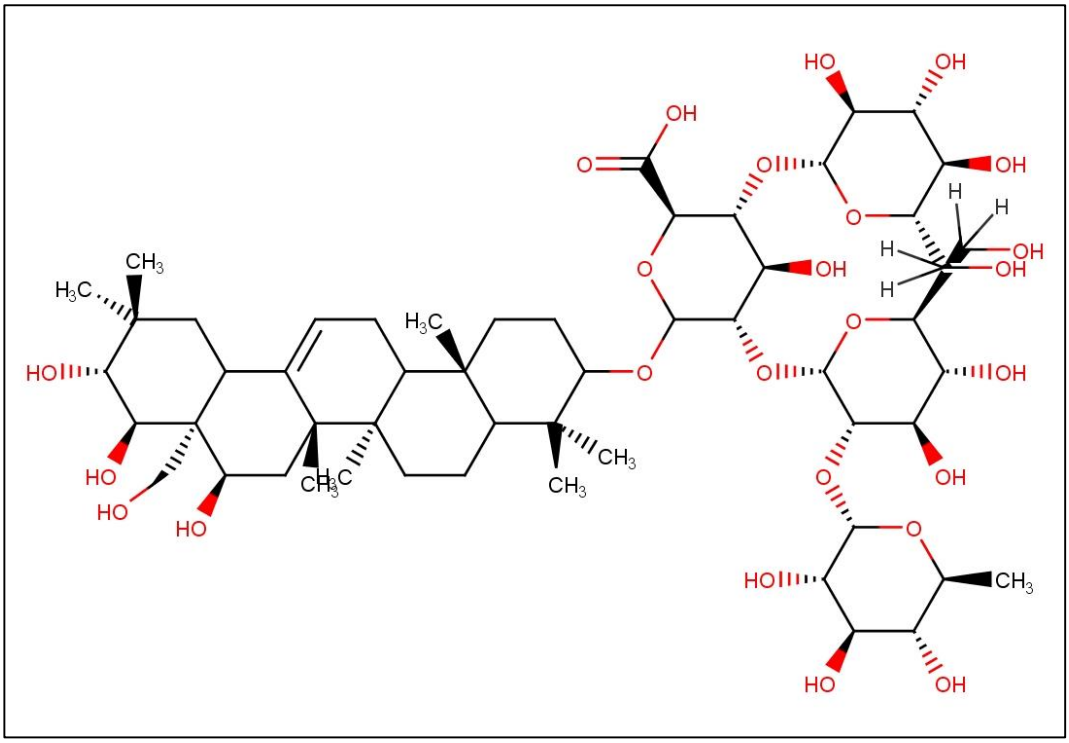


Figure 6: Saponin D

1.3 NATURAL EMULSIFIERS AND CHARACTERIZATION

As already mentioned, emulsifiers are surface-active substances that act through adsorption on the separation surface(s) of two phases [35]. Emulsifiers can be classified based on different criteria. They are most commonly distinguished by the charge their polar heads carry which can be cationic, anionic or nonionic. The choice of a particular emulsifier for a specific application is based on its partitioning properties such as the hydrophilic-lipophilic balance (HLB) described by Griffin et al. [36] or their logarithmic partition ratio in octanol/water (logP). These two parameters measures the ratio of the affinity of a compound towards organic and aqueous media respectively.

The recent development of emulsifiers was driven by the processed food industry of the 20th century, which needed shelf-stable products for distribution through mass-market channels. Natural emulsifiers are slowly gaining attention from industrials, as part of the "Clean Label Movement", which aims towards replacing E-Labeled additives that might have adverse health effects. Detailed knowledge of the physical chemistry of emulsions is best obtained when pure oil, water, and emulsifiers are used [37]. According to Stoke's law, the particle size of the dispersed phase is influential to the stability of an emulsion system [38]. In this sense, the emulsion with higher resistance and control to creaming should contain particles that are small in size and homogenously distributed.

Dynamic Light Scattering (DLS) is a well-known (sub)micron particle size analysis technique [39]. DLS measures the intensity of laser light when scattered by molecules in solution. The scattered signal is detected at different angles by three different receptors (front, side and backscattering). Parameters that can be extracted

from the light scattering experiment include the translational diffusion coefficient:

$D = \frac{k_B T}{6\pi\eta R_H}$ *(Stokes-Einstein equation)* with:

D: *Translational diffusion coefficient [m^2/s] – "speed of the particles"*

K_B *Boltzmann constant [m^2kg/Ks^2]*

T *Temperature [K]*

η *Viscosity [Pa.s]*

R_H *Hydrodynamic radius [m]*

The Stokes-Einstein equation relates between the speed of the particles and the particle size. The speed of the particles is given by the translational diffusion coefficient D that is considered solely based on Brownian motion of particles. One should note that the equation's limitation lies in the onset of sedimentation, as the upper size limit for DLS measurements. In contrast, the lower size limit is defined by the signal-to-noise ratio. The translational diffusion coefficient is determined by the decay of the correlation function (describes how long a particle is located at the same spot within the sample). A mathematical procedure relates a constant measuring of the delay times and plot them into a logarithmic time axis. Cumulant algorithms are then used in order to fit the correlation function, from which D and k_B are determined. This ultimately leads to conclude R_H (hydrodynamic diameter , 'particle size') from the equation above [40].

This allows for the Z-average size of oil droplets in the case of O/W emulsions to be measured. With this said, stable emulsions have relatively low numbers of size variations, which is the so-called particle size distribution (PSD).

Another important factor that affects the stability and physiochemical behavior of emulsion is the electrical charge on its droplet surface [41]. The magnitude and sign of the charge on the dispersed emulsion particle often determine the nature of the

interaction between the droplets and other charged species nearby [41]. This directly affects the repulsion between droplets influencing aggregation phenomenon. ζ-potential (Zeta potential) determination is a significant characterization technique to determine the surface charge. During a zeta analysis, an external electric field is applied and the particles travel toward the electrode that has a charge opposite to that of the particle. Particle velocity is measured by laser Doppler velocimetry, and their electrophoretic mobility is calculated from which the zeta value (in mV) is deduced [42, 43].

Also, oil-in-water emulsions are major contributors in modern food industry [44]. But oxidation of lipids in unstabilized emulsions, generates rancidity much faster than lipids in their pure form. Therefore oxidative stability of emulsions are primary for evaluating shelf-life and storage [45]. However, because many factors are related to the oxidation of lipids in O/W emulsions, it is not easy to assess the effect of oil droplet size. Some contradictory results have been reported regarding particle size effect on particle oxidation rate [46]. On one side, reports claiming that lipids in smaller oil droplets (D_{32}＝0.4-8.5 μm) are more prone to as shown by two factors. One based on higher consumption of oxygen and the formation of conjugated dienes [47], the other based on measurement of hydroperoxide and hexanol formation [48]. On the other side, other works reported a higher stability for smaller oil droplets also based on the measurement of hydroperoxide formation (for oil droplets of D_{32}＝0.8-9 μm) and the absorbance due to conjugated diene, respectively [49, 50]. What is certain is that microencapsulation of lipid particles by an emulsifier greatly improves oxidative stability. The hydrophobic moiety is an unoxidizable or hardly oxidizable component such as a saturated acyl group.

The Rancimat method is an accelerated ageing test where air is conducted through the sample in the reaction vessel at a constantly increased temperature. The induction time determined by the Rancimat is a practical and more reliable substitute to the cumbersome AOM (Active Oxygen Method, American Oil Chemists Society Surplus Method Cd 12-57) (recently declared obsolete by the AOCS Uniform Methods Committee) [51, 52] . Induction time indicates the time in hours until secondary reaction products are detected. Standardized oxidation parameters can simulate long-term storage effects by extrapolation and is widely used in regulated food industries.

1.3.1 GUM ARABIC – NATURAL EMULSIFIER

Gum Arabic (GA) is the best known natural emulsifier. Acacia Gum (GA) is the dried gummy exudate from stems and branches of trees of various species of the genus Acacia [53]. It is a biopolymer composed of mainly two polysaccharide components in accordance with the molecular weight: high-molecular-weight glycoprotein, with 90% carbohydrate, and low-molecular-weight heterogeneous polysaccharide sugars [54]. The most recent description of the gum was proposed, as consisting of a bulk fraction composed of polysaccharides moieties, a fraction of protein-polysaccharides conjugates and a smaller fraction of glycoproteins. The unique composition of gum Arabic leads it to be used widely in both the food and nonfood industries [55]. In 2014-2016, average yearly global production of GA reached 104 000 tons [56]. GA is mainly used as stabilizer, thickener and humectant in food applications such as beverages, confections and frostings, dairy product, soft candy, gelatins, puddings, and fillings [53]. It was first admitted that the proteinaceous materials of the gum are responsible for its emulsifying

properties : the treatment of the gum with a protease inhibited its emulsifying activity [57]. But in a later study [58], adsorption kinetics did not indicate a correlation between interfacial tension and the protein content of Acacia Gum. Also no direct links were observed between the emulsifying capacity of a gum sample and its nitrogen content. Therefore, the exact molecular moeity responsible of the emulsifying properties of GA is still debatable. As for the emulsifying mechanism, it is suggested that the proteinaceous moieties, among other, would unfold in order to be adsorbed at the interface and that carbohydrate groups extended into water to provide steric repulsion [59]. It it sepecifically used in beverages containing small amounts of oil or oleoresins as GA would disperse oil-based flavors very efficiently. No natural emulsifier matches GA in terms of potency and versatility [60]. We chose GA in this work as the top reference for a comparative study to show how a developed Styrax extract would line up in terms of emulsifying potency.

1.4 FRAMEWORK FOR QUANTITATIVE EXTRACTION OF SAPONINS

1.4.1 SOLVENT EXTRACTION OF PLANT SECONDARY METABOLITE

The basic principle is to grind the dried plant material in order to increases the surface area for a more efficient extraction. Earlier studies reported that solvent to dry sample mass ratio of 10:1 (v/w) is considered as ideal. Since nearly all of the identified antimicrobial compounds from plants are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction [61]. Also, two or more extraction cycles are needed with an average time of 9 hours each [62].

1.4.2 AB-8 Macroporous Resin Adsorption Chromatography

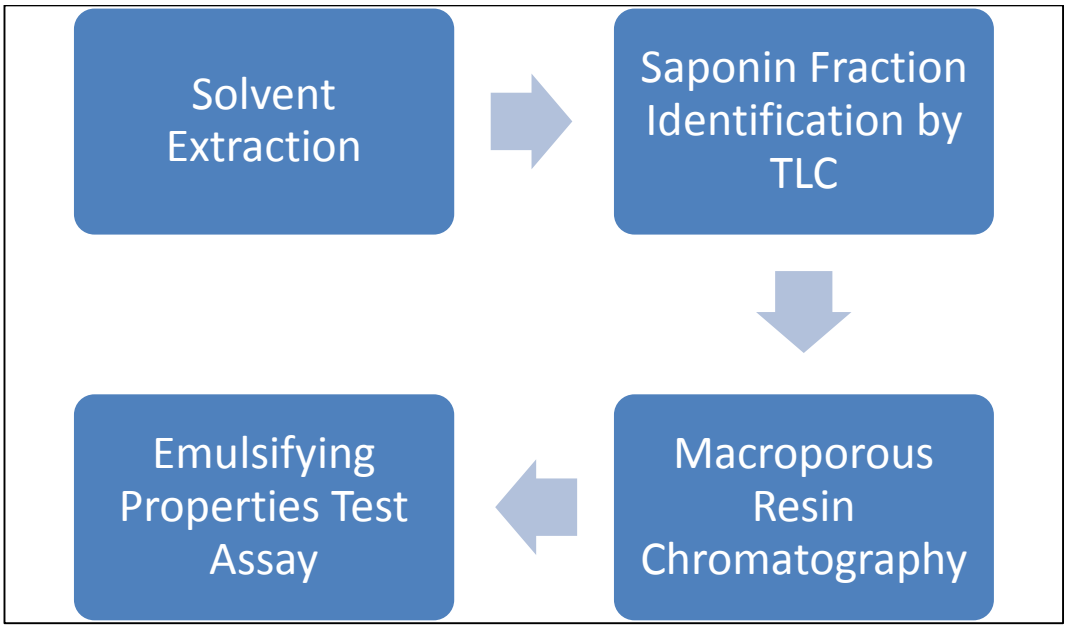
Recently, sorbents, particularly macroporous resins, have attracted increasing research attention in enriching bioactive compounds from extracts of raw herbal materials [63]. Macroporous resins can selectively adsorb targeted constituents from aqueous and non-aqueous systems through hydrogen-bonding interaction, van der Waals force (hydrophobic interaction), complexation, size-sieving action, and electrostatic force and can achieve separation based on differences of adsorbent affinity toward adsorbate molecules [63]. Considering their moderate purification effect, stable structure, high adsorption capacity, low operating cost, low solvent consumption, long service life, and feasible regeneration [64], macroporous resins have been widely applied for separating and purifying glycosides, carotenoids, fatty alcohols, flavonoids, alkaloids, and other active ingredients extracted from plant materials. Yang et al [65] tested different hydrophobic resins and concluded that the most performant was of the type "AB-8" (crosslinked polsyterene) for it has the best desorption speed/efficiency towards saponins as tested on Tea Seed Saponins of *Camellia Oleifera*. Yang also determined the optimal flow rates used in loading/washing for maximum adsorption/desorption of the used resin. The principle behind this particular technique regarding sample purification, is to eliminate extremely hydrophilic (polysaccharides) and hydrophobic (flavonoids, pigments etc.) compounds so we can afterwards collect the "middle", neutral fraction where saponins are found. One should note that the pore size of the AB 8 resin is 140 Å on average, while alkaloid and steroidal saponins are well above 150Å.

1.4.3 TLC Colorimetry for Determination of Saponins

Following the concentration of the crude extract, TLC spray colorimetry is applied to identify the saponin fraction so it can be kept track of and make sure that the preliminary sample purification is successful. TLC colorimetry is a simple 15 minutes application where different fractions from the sample migrate on silica gel that is immersed in a specific solvent (eluent). The fraction will be identified by a spray reagent that will specifically react with the target fraction to give a distinguishable color. The Vanillin-Sulfuric assay is a reliable protocol specific to saponins [66] [67] (see Section 2.4 for detailed protocol).

CHAPTER 2 : MATERIALS AND METHODS

Conventional separation is usually performed by solid–liquid extraction from raw resources, followed by precipitation using different solvents; the entire process is cumbersome and the products have a dark luster color, lack of texture, and low purity [65]. Therefore we have developed a protocol that is conceived for a straightforward isolation of secondary metabolites that are difficult to extract using less steps and solvents required while achieving the maximum efficiency and selectivity.



2.1 MOLECULAR MODELING USING MARVIN SKETCH SOFTWARE

Following the investigation on the *S. Officinalis* saponins, the only structural information available was the one offered by Yayla et al. [5]. No information was found online on PubChem nor ChEMBL (database of chemical compounds) on *Styrax Officinalis* saponins. This initiated the move here toward using software

simulation to integrally and manually “draw” each saponin in order to render their 3D structure that will allow better understanding of their physical properties. For this purpose Marvin Sketch version 19.19 was used and exclusive novel data was gathered including logP partitioning and HLB of each of the 4 saponins.

2.2 SAMPLE PREPARATION

Styrax fruits were collected on August 29 and September 10 2019 from the region of Kesserwan, Lebanon (Eghbe, Balloune and Ain-El-Rihane). Samples were identified as *Styrax Officinalis* by Dr. Tanos G. Hage Associate Professor of Biology at the Dept. of Sciences, FNAS, Notre Dame University – NDU. Samples are kept in the NDU chemistry lab. The Pericarps were deseeded and oven dried @ 60^{o}C for 48h and pulverized using a local mill from the NDU Engineering Laboratory (Fig. 9).

Figure 7: Styrax Officinalis fruits from the region of Ballouneh, Mount Lebanon

200g of dried plant powder were extracted in 2 cycles of 24h each, using 2 Liters

of 70% ethanol per cycle. The extract was filtered and concentrated to 650mL of Crude Extract using a rotary evaporator.

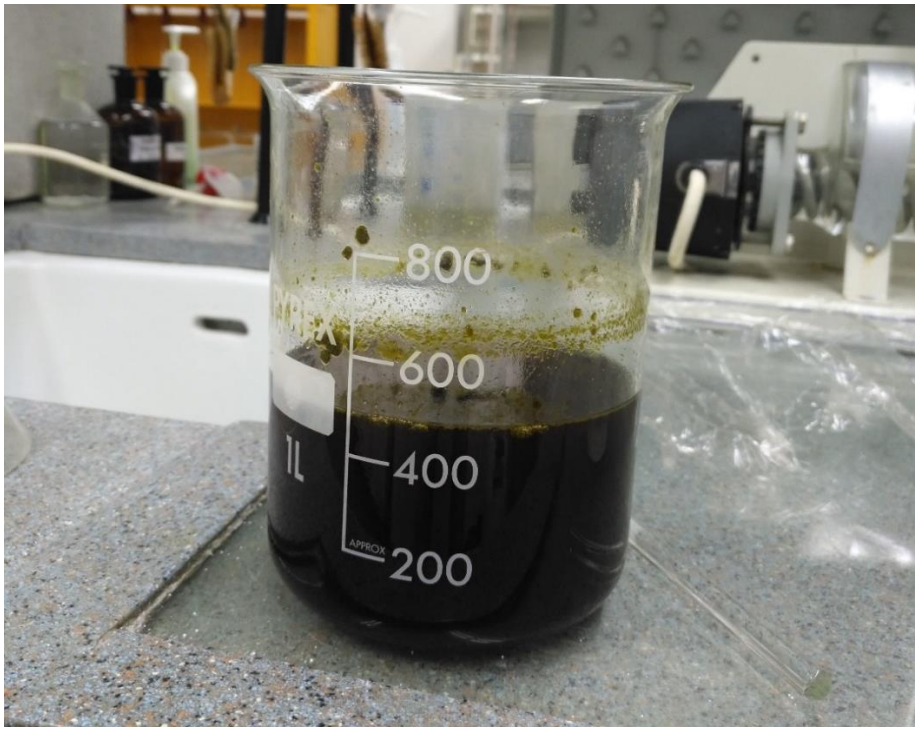


Figure 8: Concentrated Crude Extract

2.3 SAMPLE PURIFICATION USING ADSORPTION CHROMATOGRAPHY

70g of dry AB-8 Macroporous Resin (obtained from Anhui Sanxing Resin Technology Co., Ltd, China) were pretreated by washing with 5% NaOH followed

Figure 9: Concentrated Saponin-Containing Ethanolic Fraction

by 5% HCl to remove potential impurities related to the manufacture of the material (monomers, heavy metals, impurities etc.). The resin was then soaked for 24h in 95% ethanol and washed with deionized water for subsequent filling of the column. A 70cm by 2.4cm fritted column was used for the adsorption chromatography and was packed using the wet packing technique [68] with a Bed Volume (BV) equivalent to 103mL. Flow rate was set to 2 BV/h. The column was first loaded with 5 BV of Crude Extract. First elution was made using 9 BV deionized water in order to eliminate hydrophilic contents such as sugars and cellulosic compounds. Washing with 0.25% of NaOH followed was performed to remove pigments, flavonoids, heterocyclic compounds and other impurities. Once NaOH effluent was colorless (10 BV), 90% ethanol (~ 5 BV) was used to desorb the Saponins. Most mentioned experimental parameters for the purification were adopted from Yang et al. [65] and further modified based on the saponins' chemical properties and solvent availability. The final ethanolic fraction was further concentrated into 250mL amorphous extract.

2.4 IDENTIFICATION OF THE SAPONIN FRACTION USING TLC COLORIMETRY

The most frequently used solvents for TLC performed on silica gel include different proportions of chloroform–methanol–water. The ratios opted in this case was a (13:7:1) $CHCl_3$–MeOH–H_2O determined by comparison of *Styrax Officinalis Sap.* to structurally similar saponins that have been previously isolated and colorimetrically treated. The assay used here is known as the Vanillin – Sulfuric Acid method also reputable for being the most stable with consistent coloring [67]. After TLC, the plate is sprayed with 10mL 5% ethanolic sulphuric acid followed

immediately by 10 mL of 1% ethanolic vanillin (98% purity, obtained from Bio Basic Inc. Lab Equipment) and then heated at 110^{o}C for 5-10 minutes. Saponin-containing spots will give blue to blue-violet coloring.

Figure 10: Positive TLC colorimetry of the final ethanolic fraction in triplicate.

2.5 EMULSIONS PREPARATION

Oil-in-water metastable macro emulsions were prepared with food grade sunflower oil at 10% w/w (of water) and emulsifiers (Gum arabic and Styrax extract) at 4 concentrations (2.5, 5, 7.5 and 10% w/w of total solution). Acacia gum of analytical grade was obtained from Paskem Finechemicals Industries Inc. The organic phase was prepared by adding emulsifier to the oil while stirring at ~ 200rpm for 5min. The aqueous phase (deionized water) was added and the mixture homogenized at 1200rpm for 20min. Emulsions were then subjected to sonication using Fisherbrand® FB11021 Ultrasonic Bath @ 35 KHz for 20min and homogenized again at 1200rpm for 10min. to ensure reliable result.

2.6 CHARACTERIZATION OF PRIMARY EMULSIFYING PROPERTIES

2.6.1 PARTICLE SIZE

Measurements were done in triplicates using Zetasizer Nano ZSP from Malvern Inc. and operated with the provided software. Samples were diluted 10 fold for all DLS measurements. The parameters set to determine PS and PDI are mentioned in table 1. All samples were taken from freshly prepared emulsions.

Sample Size	Dispersant	Material	Cell Temperature	Cell Type
800 µL	Water	Sunflower Oil	25 ^{o}C	Disposable Cell – DTS0012

Table 1: PS determination parameters

2.6.2 ZETA POTENTIAL

Particle Zeta Potential measurements were performed with the same equipment mentioned in the previous section (2.6.1) and determined in triplicates using the below parameters. All samples were taken from freshly prepared emulsions.

Dispersant	Material	Temperature	Cell Type
Water	Sunflower Oil	25 ^{o}C	Folded Capillary Cell

Table 2: Zeta-Potential determination parameters

2.6.3 OXIDATIVE STABILITY

Emulsion Oxidative Stability was measured using Rancimat model 743 by Metrohm Inc. and operated using the provided software. A pure SFO sample (4.5g) was first tested as a reference value. As for both Gum Arabic and Styrax-stabilized emulsions, 6g samples were used from freshly prepared batches. Method parameters are mentioned in the table below.

Temperature	Gas Flow	Delta T	Stop Criteria	Evaluation Criteria
80 ^{o}C	20 L/h	1.1	Endpoint	Induction Time (hours)

Table 3: Rancimat method parameters

CHAPTER 3 : RESULTS AND DISCUSSION

3.1 INFORMATION GATHERED BY SOFTWARE SIMULATION

The different information acquired relevant to this study are included in a comprehensive table (table 4). A 3D structure of each of the 4 triterpenoid saponins occuring in the extract (A, B, C and D, as identified by Yayla [5]) was rendered (figure 11 to 14). The simulated structures show a 'mushroom' shaped geometry in common for the four saponins. A first glance on the M_W indicates that all four Styrax saponins are middle-sized, knowing that saponins M_W ranges from 700 D to 1900 D. Figure 11 to 14 show the saponins' individual log P distribution (and polar surface area) in aqueous media. Note that all 4 saponins have log P close to 0 (with very little hydrophilic tendency) and HLB (Griffin) [36] close to 10. These values backs up the fact that these molecules are particularly amphiphilic and encourage further investigation on structure-related surface rheology. Also our choice of testing on O/W emulsions is based on these particular characteristics, including the choice of gum Arabic (HLB 8~10) as a reference emulsifier for comparative studies. Additional structural details and properties (pKa, polarizability, refractivity, polar surface area…) were also extracted but are not of current relevance to this work.

Saponin	Molecular Weight (D)	logP	Hydrophilic-Lipophilic Balance (HLB)
A	1260	- 0.32	10.12
B	1283	- 0.04	9.95
C	1275	0.17	10.01
D	1137	- 2.97	10.28

Table 4: Styrax saponins structural properties

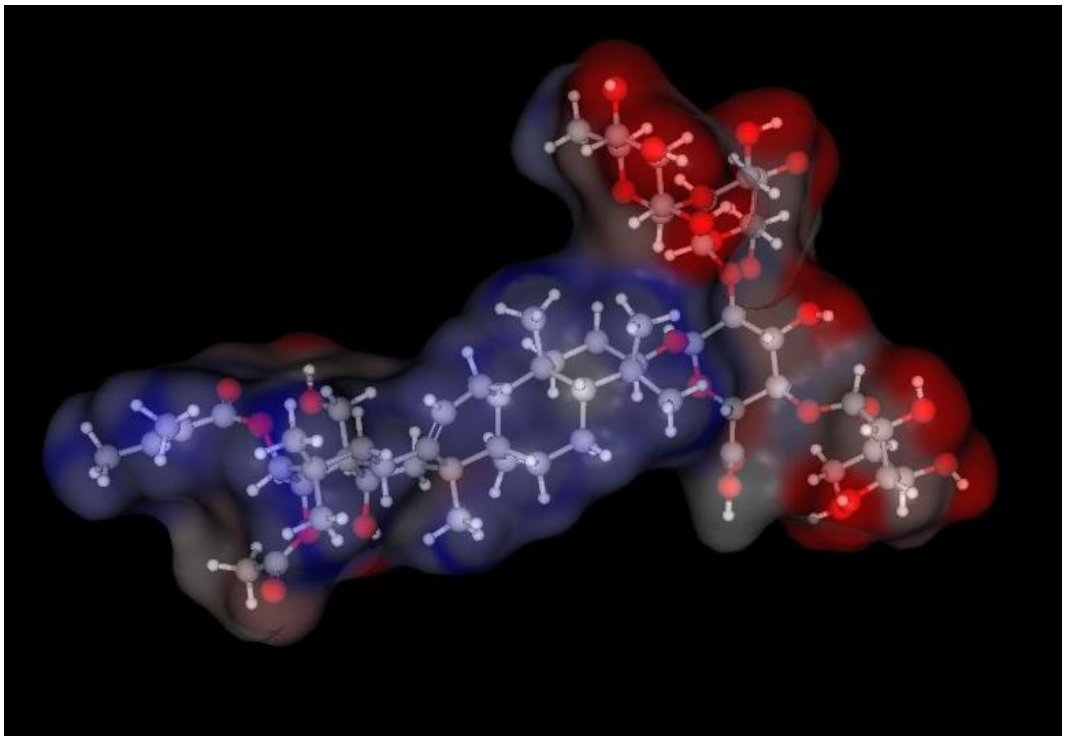


Figure 11: Saponin A

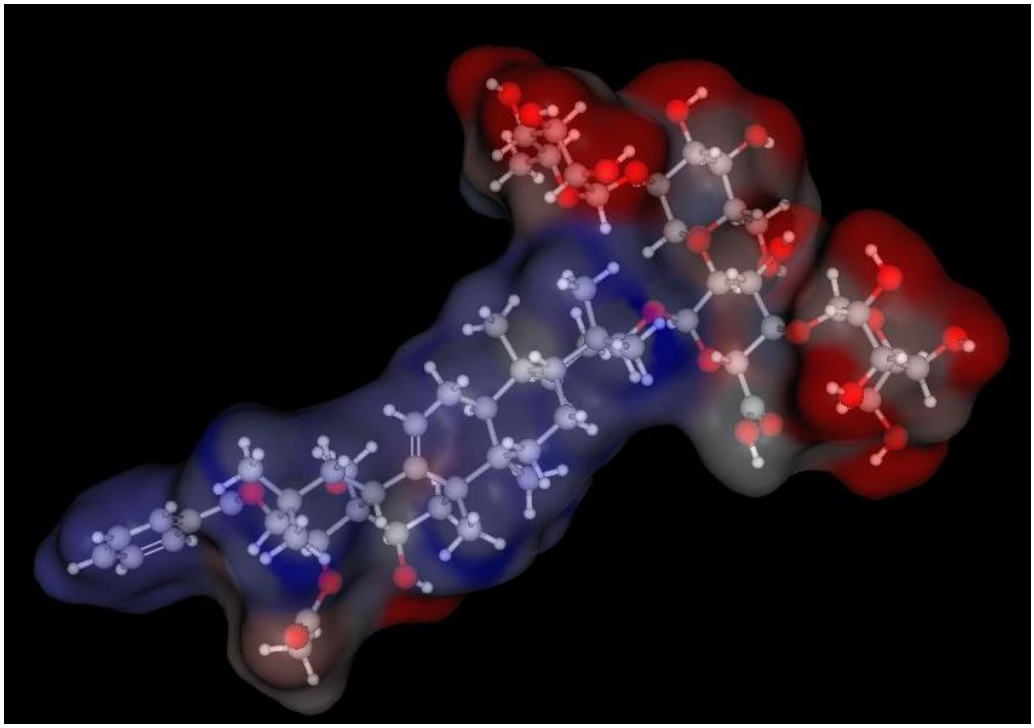


Figure 12: Saponin B

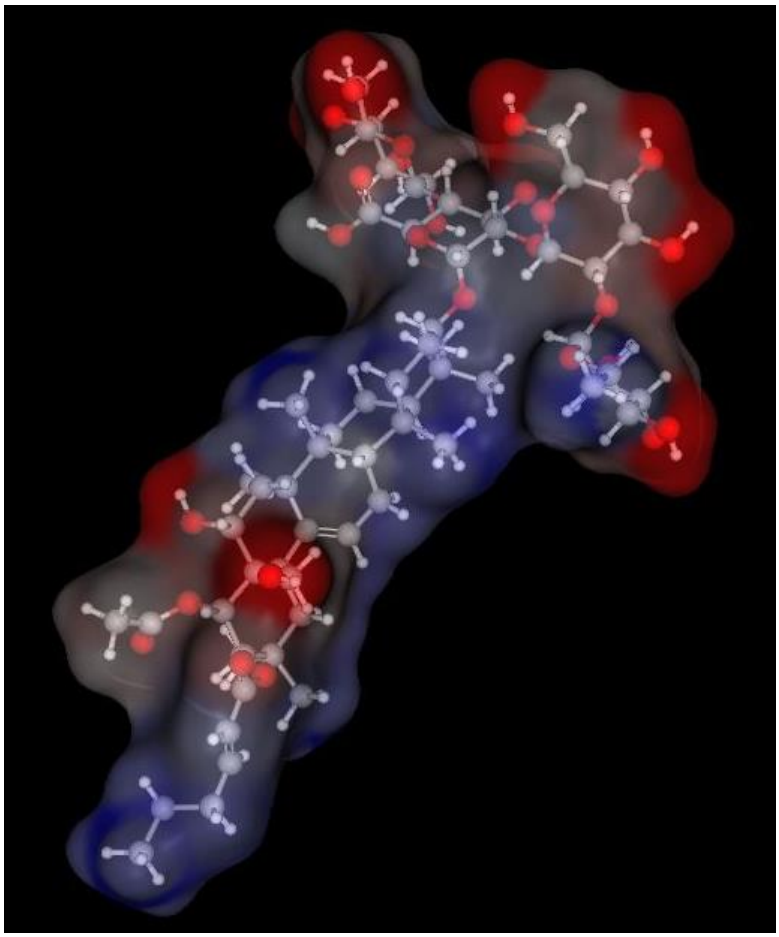


Figure 13: Saponin C

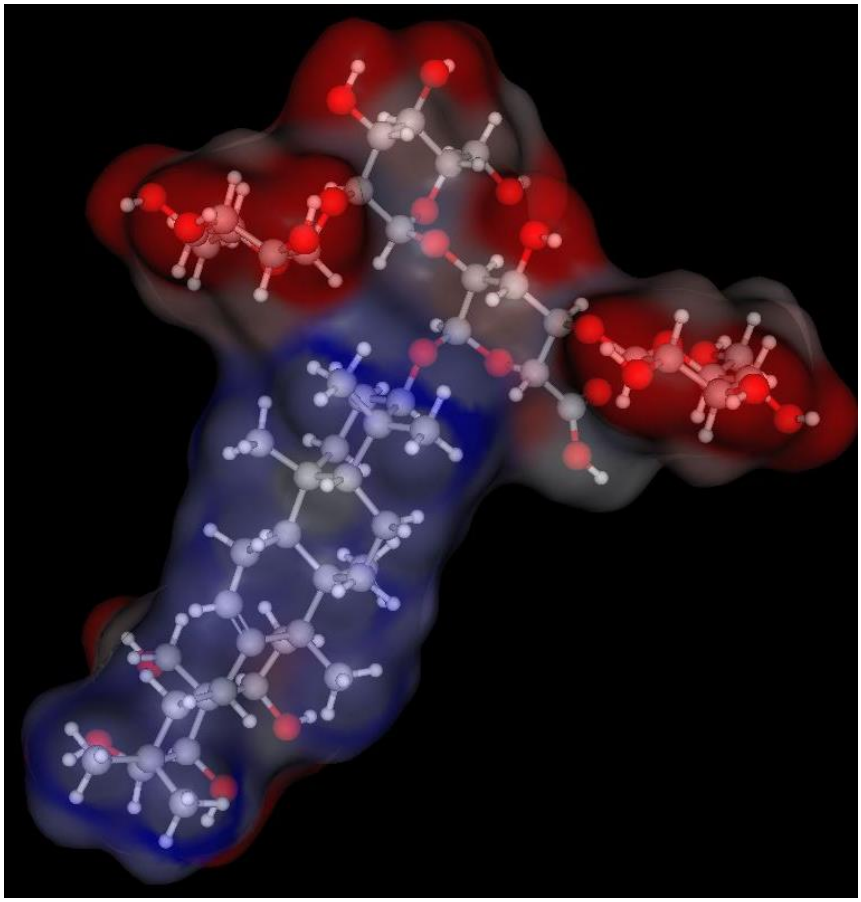


Figure 14: Saponin D

3.2 COMPARATIVE STUDY ON EMULSIONS STABILIZED BY STYRAX SAPONINS EXTRACT AND ACACIA GUM

Styrax stabilized emulsion were compared to emulsions stabilized by gum arabic (acacia gum) as they both have similar hydrophobic/hydrophilic partitioning ratio (logP and HLB, see section 3.1) and the latter being of industrial and commercial importance. Below is a table containing the particle sizes and PDI of the different emulsions at different emulsifier concentration.

Emulsifier Concentration (%, w/w)	Acacia Gum Emulsion			Styrax Emulsion		
	PS(Z-avg, nm)	PDI	ζ-potential (mV)	PS (Z-avg, nm)	PDI	ζ-potential (mV)
2.5	5382	0.012	-22.5	549.7	0.476	-91
5	3793	0.177	-12	352.4	0.440	-84
7.5	1951	0.172	-15.1	365.9	0.379	-81
10	1691	0.025	-10.5	318.2	0.493	-94

Table 5: Styrax vs Acacia Gum PS, PDI and ζ-potential

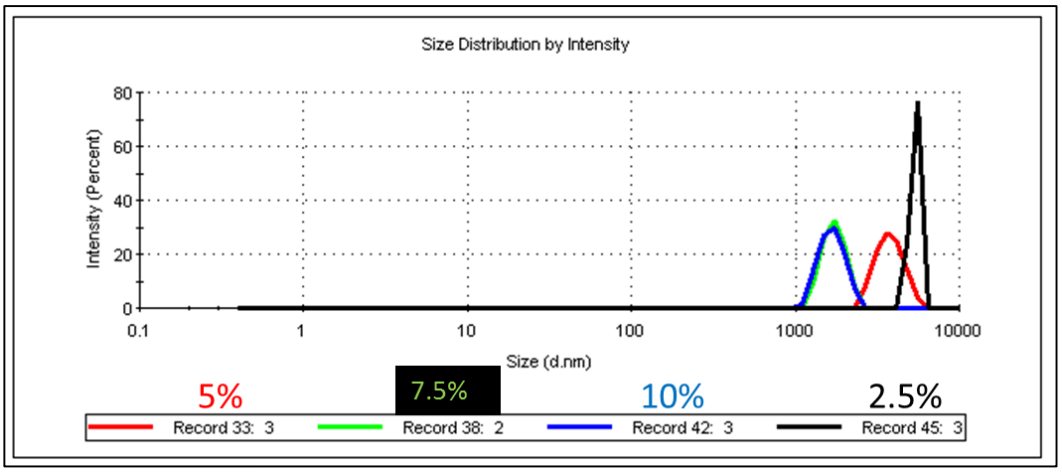


Figure 15: Size distribution by intensity from DLS of the Acacia Gum emulsion PS at different concentrations.

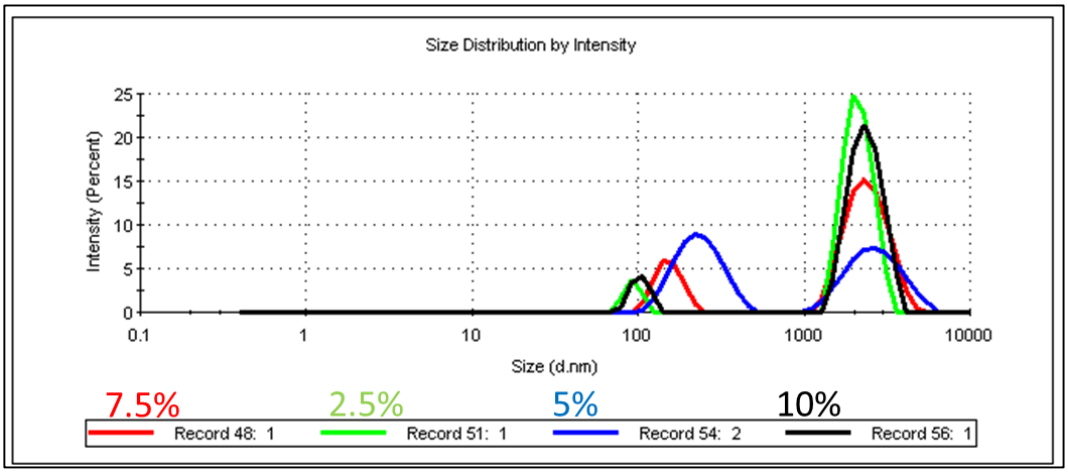


Figure 16: Size distribution by intensity from DLS of the Styrax emulsion PS at different concentrations.

The common trend observed between the two emulsifiers is the decreasing particle size Z-avg with increasing emulsifier concentration as oil particles are being better encapsulated. Interestingly, Styrax stabilized emulsions has much smaller particles (318 ~ 549 nm) than their Acacia Gum (1691 ~ 5392) counterpart. Contrarily, Acacia Gum emulsions had lower Polydispersity Index (PDI) than the Styrax emulsions, meaning a narrower size (and M_W) distribution along the emulsion. Evidence as in Figure 15 show the narrow intensity peaks of acacia gum and the broader peak distribution of Styrax extract in figure 16. These can be rationally explained by the fact that the Acacia gum powder used is of analytical grade i.e high purity (98%) and exact composition (high molecular weight polysaccharides of same or close M_W). The Styrax emulsion on the other hand showed bimodal size distribution. This could be due to the mixed nature of the saponin extract fraction. Four different saponins of different molecular weights (among others) (see section 3.1) could result in different sizing of droplets. As for the size differences between the Acacia and Styrax emulsions, the results could be directly related to their different binding mechanisms. Arabinogalactan protein complexes of acacia gum adsorb to the interface with the polysaccharide moieties protruding into solution hence the gum's emulsifying ability is believed to occur mainly by steric repulsion [69]. In the case where high diffusion rates (smaller particles by better pre-homogenization) are achieved with the gum, higher oil/water contact surfaces make way to amino acid groups to be relatively more available. This can drastically lower particles surface tension and increase emulsion stability [70]. In the case of macro emulsion, arabinogalactan proteins do not entirely protrude therefore particles are not as charged as in nanoemulsion. This also explains the moderately charged to

neutral particles of acacia emulsions in table 5. Golkar et al. tested gum Arabic on soybean oil (similar in composition to sunflower oil used here) emulsion [71]. The ζ –potential results of GA emulsions (-10 mV ~ -22mV) in this study aligns well with Golkar's works (-30mV) as forming moderately charged particles. Note that the Z-avg of particles is our case are much smaller. Also worth mentioning is that GA has different binding affinity to oils having different FA compositions resulting in different emulsifying powers [72]. This can prove the legitimacy of our work and it's comparison to Golkar et al.'s study by using an oil with similar composition. As for the Styrax emulsifier, one should emphasize that it is an ethanolic extract, and ethanol is known for breaking emulsions. Outstandingly, even with the presence of this emulsion breaker, the Styrax extract proved to have very potent emulgator effect. Many explanations can be provided to explain the emulsification properties of *Styrax Officinalis* saponins extract. In terms of molecular structure, these saponins have two different moieties that are well distinguished marking their distinctive amphiphilic nature. Moreover their mushroom shaped 3D structure (Figure 11 to 14), along with the above mentioned properties, suggests that these molecules anchor along the oil/water interface in an unusually stable position. These observations can back up previous works that have mentioned unique rheological properties of triterpenoid saponin adsorption layers. It was shown that triterpenoid saponins stack up at the aqueous interface through self-assembly via steric packing based on saponin shape. The structural "bend" between the hydrophilic and hydrophobic parts in triterpenoid saponins causes tilted anchoring with respect to the interface normal [9, 13]. This ultimately leads to partial submergence of the hydrophobic moiety into the aqueous phase, based on the length

of the sugar chain [13, 73]. These mechanistic behaviors confer the oil/water interface unique visco-elastic and shear resistant properties and might explain the observed particle stability in its physical or charged aspect, in the Styrax-stabilized emulsion.

3.3 OXIDATIVE STABILITY OF EMULSIONS

Induction time of the different emulsion are listed in table 6 below. It indicates time until secondary reaction products generated by the sample are detected by the Rancimat.

Emulsifier concentration (%, w/w)	Induction Time (hours)		
	Sunflower Oil (reference)	Acacia Gum	Styrax
2.5	44.46	54.61	48.9
5		43.23	48.5
7.5		9.3	36.4
10		20.5	54.5
	Average:	31.91	47.075

Table 6

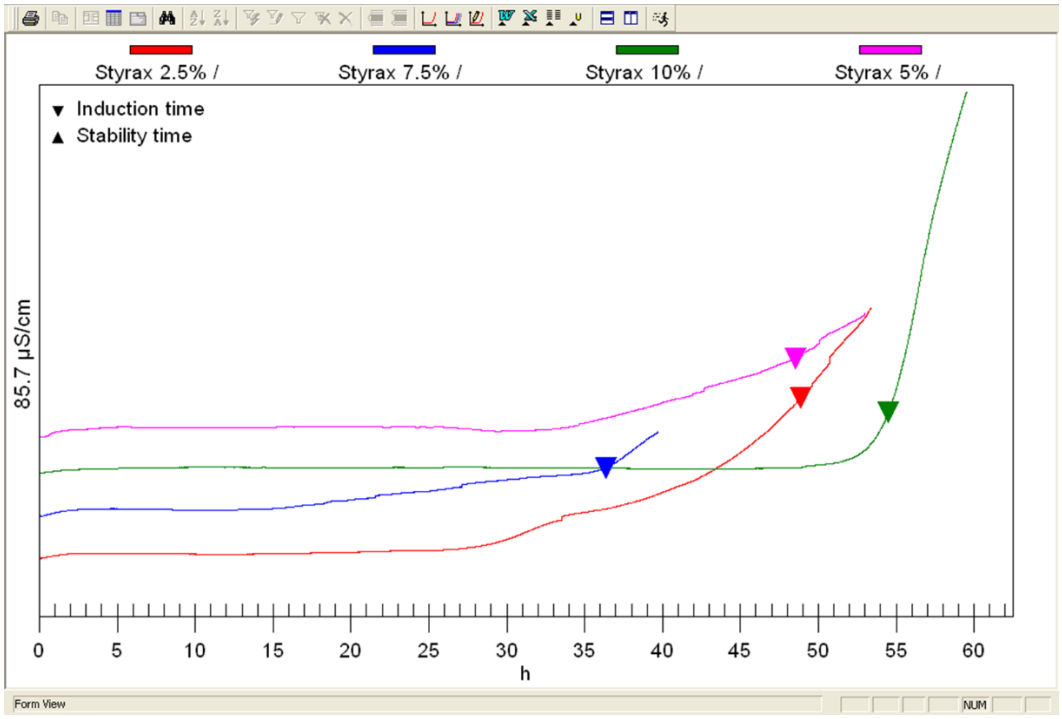


Figure 17: Graphical determination of the induction time of Styrax emulsions.

Many studies have shown that lipid oxidation in O/W emulsions is influenced by the emulsifier charge. Mei et al [74] reported that emulsions made with anionic emulsifiers were subject to higher oxidative stability than emulsions made with neutral or cationic emulsifiers. With this said, Styrax stabilized emulsion (having more negative particle charges) did showed an average induction time (47.075 min) longer than the reference oil (44.46) and acacia stabilized emulsion (31.91 min). Thus, the Styrax emulsion having the longest average induction proves to have better droplet encapsulation. Hypothetically these results line up with the suggested hypothesis on particle charge. However, a closer look at the results indicates very little or no correlation with respect to the emulsifier concentrations. One of the Styrax emulsions (7.5% w/w extract) showed a lower stabilization than of the oil reference, and one of the Acacia Gum emulsions (2.5% Acacia w/w) showed longer induction time contrary to the other concentrations. With this said, the results trend

for both emulsions is not clear to consider a definitive conclusion explaining emulsifying behaviors of both emulsifiers. More extensive testing on oxidative stability is needed to clearly assess reliable results leading to a more definitive conclusion. What is known based on the current numbers is that the Styrax extract showed successful encapsulation of oil droplets in 10% O/W emulsion.

CHAPTER 4 : CONCLUSION

This study generated novel structural and physical properties of *Styrax Officinalis* saponins and successfully modified and applied an extraction protocol valuable for preliminary testing and characterization of saponins. Adsorption chromatography using AB-8 resin proved to be a practical and efficient pre-purification process that can act as substitute to other cumbersome methods of purifying crude plant extracts. Colorimetric TLC was successfully used to identify saponin presence in the pre-purified fraction by the Vanillin – Sulfuric Acid method. Dynamic Light Scattering results showed that Styrax saponins extract formed smaller particles in the 190-900nm range but had broader size distribution (PDI ~ 0.4) than Acacia stabilized emulsions (1000-5000nm range, PDI ~ 0.01). Particles of the Styrax emulsion also showed higher stability through lower ζ-potential (-90 mV) than Acacia gum emulsions (~ -15mV). Oxidative stability results for both emulsions were inconclusive but Styrax emulsions showed more stability compared to the reference sample of pure oil. Lastly, the results from this work, compiled together lead to the

conclusion that saponins, including the *Styrax Officinalis* saponins, are definitely subject of great interest and deserve an integral study on both quantitative isolation and in-depth physical characterization. The proven emulsifying potency of saponins might also be related to its unique interfacial behavior paving the way for promising applications in commercial and medical fields.

CHAPTER 5 : FUTURE PROJECTIONS

Based on our preliminary work and promising results, saponins from *Styrax Officinalis* are worth a thorough investigation on their physio-chemical properties. Among the work that should follow, is the quantitative analysis of the saponin content of *S. Officinalis* following HPLC isolation of the saponins. This will allow the saponins to be eligible for testing on certified assays and regulations specific for the field of application. Worth mentioning is that *S. Officinalis* saponins are already patented (2018) in Russia for synthesis and use as spermicide under patent RU2686716C1. Also the same aglycone isolated by Anil [8] is patented in the US (patent US20070270375A1), Japan (patent JP4790620B2) and New Zealand (patent NZ547377A) as analgesic classified under 'Polyterpene Radicals'. This proves that saponins are slowly but steadily taking pace into being administered in the market.

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LIST OF ABREVIATIONS

BV = Bed Volume

D = Dalton

DLS = Dynamic Light Scattering

HLB = Griffin's Hydrophilic/Lipophilic Balance

M_W = Molecular Weight

logP = Logarithmic Partitioning ratio

O/W = Oil in Water

PDI = Polydispersity Index

PS = Particle Size

Sap = Saponin

SFO = Sunflower Oil

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