UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY TECH

Escuela de Ciencias Químicas e Ingeniería

TÍTULO: Preparation and Characterization of Emulsions containing Blueberries Mill Extract to be used as a Moisturizing and Antioxidant Agent

Trabajo de integración curricular presentado como requisito para la obtención del título de Química

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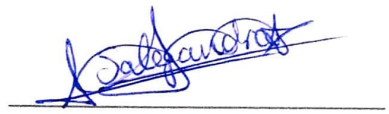

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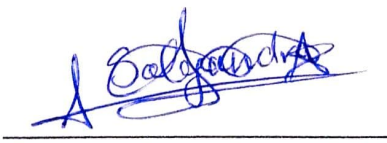

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Dedicatoria

Este trabajo está dedicado a las personas más importantes de mi vida.

A Dios, con quien me sentí cerca espiritualmente en todas las alegrías y dificultades que surgieron durante mi carrera.

A mis padres y hermanos porque siempre me mostraron su apoyo y cuidado.

A mi abuelita (+) que solo me acompañó durante mi primer año de estudios pero a quien recuerdo todos los días porque es uno de mis mejores ejemplos a seguir.

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Saríah Alejandra Barreiro Tobar

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Resumen

Los agentes hidratantes y antioxidantes son necesarios para la salud del cuerpo humano, comúnmente ayudan en la regeneración de la piel, la eliminación de puntos negros y el tratamiento de manchas causadas por factores externos, ambientales, mala alimentación y cambios hormonales. Por estas razones, la investigación del cuidado de la piel se ha intensificado a lo largo de los años, por lo que se realizan investigaciones todos los días para innovar productos existentes y desarrollar nuevos productos.

Este trabajo de investigación está enfocado en la obtención de un extracto de arándano para ser utilizado como agente antioxidante y para analizar su encapsulación en una doble emulsión de tipo Agua/Aceite/Agua (W/O/W, por sus siglas en inglés) para potenciar su capacidad antioxidante y proporcionar beneficios a la piel. Tanto el extracto de arándano como las emulsiones preparadas a partir de él se analizaron y caracterizaron. El extracto obtenido se caracterizó por espectroscopia ultravioleta e infrarroja, y se llevaron a cabo otros procedimientos para determinar su poder reductor. En cuanto a las emulsiones, estas se caracterizaron por pruebas de dilución para conocer el tipo de emulsión formada y análisis por microscopía óptica para confirmar las fases de las emulsiones. Además, se evaluó el comportamiento de las emulsiones y el extracto de arándano contra la radiación UVA y UVB.

Se evaluaron algunas propiedades exhibidas por las emulsiones preparadas, tales como su capacidad humectante y de protección solar, así como la propiedad antioxidante del extracto de antocianina. Se obtuvieron resultados satisfactorios, demostrando la capacidad de los materiales obtenidos para reducir las manchas en la piel, además de hidratar y proteger contra la radiación UV.

PALABRAS CLAVE: Actividad antioxidante, protección solar, emulsión, extracto de arándanos, arándanos, hidratación, cromatografía.

Abstract

Moisturizing and antioxidant agents are necessary for the health of the human body, they commonly help in the regeneration of the skin, elimination of blackheads and treatment of spots caused by external, environmental factors, poor diet, and hormonal changes. For these reasons, skincare investigation has intensified over the years, so research is being carried out every day to innovate existing products and develop new products.

This research work focuses on the obtaining of a blueberry extract to be used as an antioxidant and moisturizing agent, and to analyze its encapsulation in a double emulsion Water/Oil/Water (W/O/W) to potentiate its antioxidant capacity and provide benefits to the skin. Both blueberry extract and emulsions prepared from it were analyzed and characterized. The obtained extract was characterized by UV-Vis and IR spectroscopy and other procedures were carried out to determine its reducing power. As for emulsions, these were characterized by dilution tests to know the type of emulsion formed and analysis by optical microscopy to confirm the phases of the emulsions. Also, it was evaluated the behavior of emulsions and blueberry extract against UVA and UVB radiation.

Some properties exhibited by prepared emulsions, such as their moisturizing and sun protection capacity, as well as the antioxidant property of the anthocyanin extract were evaluated. Satisfactory results were obtained, demonstrating the ability of the materials obtained to reduce spots on the skin, in addition to moisturizing and protecting against UV radiation.

KEYWORDS: Antioxidant activity, solar protection, emulsion, blueberries extract, blueberries, moisturizing, chromatography.

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INTRODUCTION

In recent years, skincare has become indispensable for everybody around the world. Within a daily skincare routine, an indispensable product, always present, serves to provide extra hydration to the skin: a moisturizer. The hydration can be lost due to environmental and hormonal factors. Dehydration causes the skin to look aged over time, exhibiting features as reducing elasticity and flexibility, yellowish skin, increasing wrinkles, furrows under eyes, pigmented spots, skin shine reduction, among others.[1] All these signs are because the body does not have a stable defense of antioxidants, so damage to the skin tissue and other tissues occurs, therefore, the DNA is affected.[1]

Certain cosmetics give to the skin some beneficial substances, such as antioxidants, vitamins and minerals, necessary for counteract the free radicals causing DNA damage, also providing such as polyphenol and flavonoids, prevents these DNA damage progressively improving the skin appearance. Even more, these antioxidant compounds act as de-pigmenting agents by inhibition of synthesis of melanin, which is responsible of production of pigments in skin.

Among the natural sources of antioxidants are the fruits known as berries, which in addition to being very delicious, contain a significant amount of chemical compounds, especially polyphenolic compounds such as anthocyanin and flavonoids, with antioxidant properties, converting them in very convenient materials to be used in cosmetic formulations. To potentialize the activity of polyphenols present in these berries, it is proposed the encapsulation of their extract in the resulting emulsions. Upon encapsulation, the extract presents a better distribution throughout the emulsion, improving its bioavailability, in addition to being protected from oxidation and thermal degradation. For fat-soluble extracts, encapsulation can be carried out in an oil-in-water (O/W) emulsion, while for water-soluble extracts a double W/O/W emulsion is more convenient. In the last case, the aqueous phase containing the extract is located in the innermost part of the emulsion.[2,3]

Blueberry is a fruit belonging to berries family and is believed to have one of the highest antioxidant content of all fruits and vegetables.[4,5] In particular anthocyanins, flavonoids are the main type of polyphenols compounds present in the blueberries, and responsible for most of their beneficial health effects. Therefore, in this work has been proposed the use of the blueberry extract in the preparation of cream with moisturizing and antioxidant properties. There are several methods for the extraction of anthocyanins from blueberries, and in general, they involve the use of acidified polar organic solvents and a controlled purification process.[6]

Once the extract is obtained, its chemical and morphological characterization is required, through the convenient and available spectroscopic and microscopic analysis. Also, for the evaluation of the phenolic compounds content, antioxidant and moisturizing capacities of the extract, the respective assays were developed.

In the preparation of emulsions proposed as cosmetic products, an analysis of the respective reagents is required. The selection of components of each of three phases of a double emulsion is established, particularly the choice of appropriate surfactants, to guarantee stable emulsions and with the expected properties. After the development of emulsions and evaluation of the obtained results, some suggestions are put forth to provide information to improve the preparation method and the quality of the final product.

This document is presented as a compilation of different chapters that were carried out during the project distributed as follows. Chapter 1 presents the *Theoretical Background* to raise awareness of the fundamental concepts, Chapter 2 presents the *Problem Statement* and the *Objectives*. Chapter 3 contains the information of the *Experimental Methodology* developed to obtain the *Results* presented and discussed in Chapter 4. Finally, in Chapter 5 the *Conclusions* are presented, as well as the proposed suggestions.

1. CHAPTER 1. THEORETICAL FRAMEWORK

1.1. Antioxidants in cosmetics

When the skin damage is reached, with a dry and dull appearance, like a leathery skin, aging is accelerated with the appearance of wrinkles, dark circles under the eyes and with a decrease in elasticity and flexibility.[1] The antioxidants compounds, which are based on molecules capable of preventing or retarding the oxidation of other molecules, and therefore, prevent cell damage, have an important role in the formulation of skin care products.[1]

Antioxidants are made up of vitamins and minerals, which can counteract free radicals, which come from solar radiation, automotive pollution, industrial sources, bad nutrition, and other environmental factors, that damage DNA, lipids and proteins. Vegetables and fruits are a rich source of antioxidants, so currently; their consumption has increased, especially in women, for their direct benefits on the skin and for reducing the risk of disease. Among the most known antioxidants to combat skin problems are: vitamins (A, B, C, D, E, H), zinc, lactic acid, citric acid, salicylic acid, benzalkonium chloride, resorcinol, a plant extract with anti-inflammatory properties.[1] Both cosmetic and dermatological formulations contain fragrances, fats, and oils that self-oxidize by exposure to air, causing an unpleasant odor in cosmetic products and their chemical degradation. In order to preserve the formulations and increase the shelf life of the product, the addition of antioxidants is used as they are soluble in lipids and water.[1,7]

Antioxidants in high concentrations can act as pro-oxidants resulting in cellular oxidation (oxidative stress), protectors of both healthy cells and harmful cells (cancer cells) and cause symptoms such as nausea and headaches.[8,9] Antioxidant stops the reaction reducing the activity of free radical, donating them missing electron. Because of this, antioxidants are the only compounds able to donate electrons without becoming reactive free radicals themselves.[10] As the name implies, antioxidants are oxidation inhibitors and they can penetrate the deepest layer of the skin by using skincare products, penetrating from the epidermis, passing through dermis up to hypodermis (subcutaneous layer),[1] indicated in **Figure 1**.

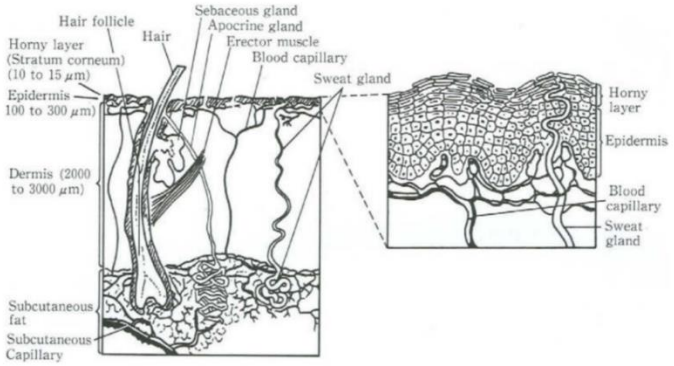

Figure 1: Basic structure of skin. Mitsui, T. (1997).[1]

Free radicals are those molecules that have an unpaired electron and it increases the chemical reactivity of the molecules. They are capable of acting in biological systems producing changes in chemical composition or cell structures which can be easily observed in the decreasing elasticity and resistance of skin.[1,10] Also, they are considered byproducts when cells burn glucose to create the energy of the body, adenosine triphosphate (ATP), for the body.[7] The loss of the electron makes the molecule electrically unstable and to regain stability, they react quickly with other substances[10] to find the electron necessary. Due to these chemical species to require an additional electron to achieve stability, free radicals oxidation chain reactions will continue occurring until an antioxidant stops them.[1,7]

Although the human body has own natural mechanism of protection against damage induced by free radicals, this ability decreases with age, whereby it is convenient the consumption or use of antioxidants which allow living cells to repair and renew; the final result is a skin that looks and feels younger.[1,7] In addition to the fact that, in cosmetics formulations, antioxidants prevent any change in the quality of the product. The effectiveness of the final product will depend on the raw materials, that is, these materials should not have impurities that promote oxidation.[1]

1.1.1. Oxidation: Oxidative Stress

Oxidation is a natural process that occurs in the human body. It consists of the transfer of an electron from one atom to another. Oxidative stress is considered as an indicator of free radical aging. The oxidation process can be generated by the interaction of matter with electromagnetic radiations, for example, the formation of hydroxyl radicals by X-ray radiation or a UV excitation generating excited states with subsequent radical formation.[1] In cosmetic products, there are two oxidation mechanisms: auto-oxidation mechanism and non-radical oxidation.

Auto-oxidation mechanism is a radical chain reaction that occurs due to the presence of 20% of oxygen in the air; it could be accelerated or inhibited by ultraviolet light, heat, metal ions, water, etc.[1] On the other hand, non-radical oxidation proceeds in the presence of singlet oxygen resulting in the ozone formation, which has an extremely powerful oxidizing capacity.[1]

1.1.2. Ultraviolet rays

Ultraviolet radiation is a predominant factor responsible for premature aging and skin imperfections, due to its daily UV exposition that causes DNA damages. When the extracellular matrix constituents of the dermis are disorganized and degraded, skin tissues are subject to inflammation. This process is called *Photosensitization.*[1,11] Wondrak et al.[11] claim that the formation of photo-excited states of skin photosensitizers is induced by the absorption of solar photons, with the subsequent generation of reactive oxygen species (ROS), organic free radicals and other toxic photo-products that mediate skin photo-oxidative stress.[11]

Most of the UV radiation, above 95%, from solar energy incident, corresponds to UVA radiation, whose wavelength is between 320-400 nm.[11] The content of UVB of sunlight, with a range of 290-320 nm, depends on the solar angle, seasons of the year and environmental conditions; around 5% of UVB radiation can penetrate the skin.[11] UVB rays are more harmful than UVA rays due to the larger amount of energy they carry. The value of the ratio between UVA-UVB rays depends on the wavelength that limits their wavelength ranges, for example, when UVA rays are from 320 nm to 400 nm and UVB from 280 nm to 320 nm, the value of the ratio of UVA-UVB content of the solar spectrum at ground level is represented by the value of 20 while the value changes to 41.3 when the wavelength limiting the range of UVA and UVB is 315 nm; both values can be taken into account according to the CIE definition (International disease classification).[12]

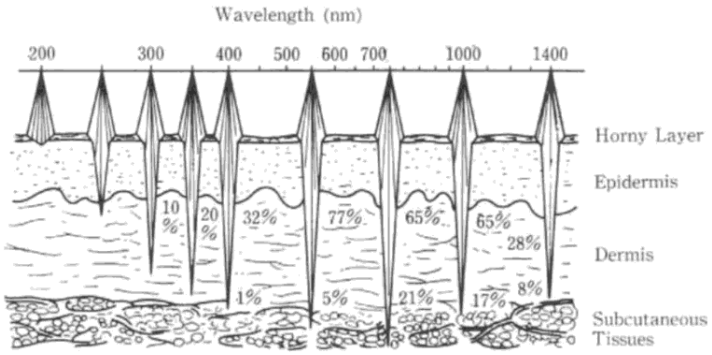

Figure 2: Skin penetration of different light wavelength. Elsevier Science B.V. (1997).[1]

A natural defense mechanism of skin against UV-light consists of absorbing and scattering it, reaching the deeper skin layer, as represented in **Figure 2**. The mechanism is developed through melanin, a pigment that provides the skin color and has a high protective power against UV rays. This is the reason by which light skins have less UV natural protection than dark skins. In some light-skinned individuals, prolonged UV exposure causes the skin to darken, as is shown in **Figure 3**.

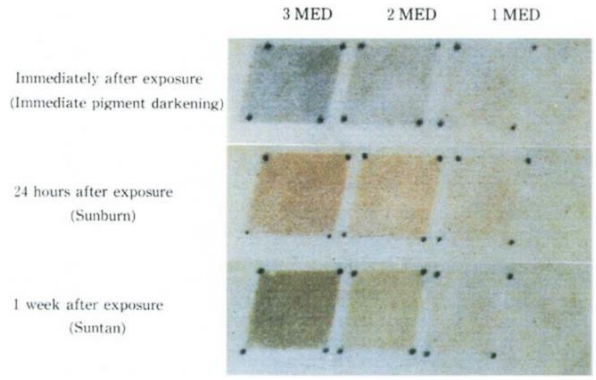

Figure 3: Changes in skin exposed to sunlight. Minimum erythema dose (MED) is a value used to indicate the acute sensitivity of individuals to UV light. Elsevier Science B.V. (1997).[1]

1.2. Blueberries

Blueberries (*Vaccinium corymbosum*[13,14]) are from the family *Ericaceae* of the order of *Ericales* and kingdom *Plantae*, they are rich in vitamin A and C.[14] The popularity of blueberries increased over the last decade, reaching 27 cultivation countries in 2011.[14] According to Food and Agricultural Organization (FAO) and the United States (U.S.A.) Department of Agriculture, U.S.D.A. is the largest blueberry producing country with an average production of over 200 thousand tons in 2013, followed by Canada (93000) and Poland (10600 tons).[14] Blueberries are processed by physicochemical procedures in the industry, this is to preserve the essential bioactivity of the fruit until it is consumed by human. The processing of blueberries is made for being used in bakery products, sauces, juices and mixtures, essences and cosmetic products. Often, the processing methods are freezing, dehydration, thermic, drying and crushing.[14]

Blueberries are soft bluish black berries of small size. The anthocyanins present in their composition are responsible for their color and some health benefits. Their structure consists of a single layer of the epidermis, without stomates, that are covered by a hydrophobic surface of cuticle and epicuticular wax, which can be seen in **Figure 4**. Epicuticular wax acts as a

protective layer against external factors (infections by bacteria or pathogenic insects, chemical agents and influence of climatic conditions) and controls the uptake of water and chemical substances into the fruit.[14]

Figure 4: A cross-section of blueberry fruit. Taken from Michalska, A. & Lysiak, G. (2015).[14]

1.2.1. Chemical composition of blueberry fruit

The chemical composition of blueberries consists of carbohydrates (sugars), amino acids, fatty acids, and a huge proportion of water (**Table 1**). This fruit has a slightly acidic taste and is a rich source of vitamin C (ascorbic acid). For every 100 g of blueberries, there is 100 mg of vitamin C.[14] Some scientific reports establish that blueberries represent a rich source of polyphenols, including flavonoids, phenolic acids, flavonols, procyanidins, and others.[14] Within biologically active compounds are anthocyanins, with the content of 495 mg/100 g of blueberries which can vary depending on ripening stage and climatic conditions.[14] These anthocyanins can be absorbed through the skin, located directly under the epidermis, protecting it from oxidative processes.

Table 1: Chemical composition of blueberries. Taken from Michalska, A. & Lysiak, G. (2015).[14]

Composition	%
Water	84
Carbohydrates	9.7
Amino acids	0.6
Fiber	3-3.5
Polyphenols	0.3
Fatty acids	0.4

1.3. Flavonoids

Flavonoids are natural pigments present in vegetables and fruits, consisting of phenolic substances, including more than 8000 known compounds.[7] They act as antioxidants due to their ability to prevent the free radical formation and protect plants from UV radiation from sunlight. The basic flavonoid structure is the flavan nucleus, shown in **Figure 5**, which consists of three arranged rings (indicated in the figure as A, B, C). Differences in the level of oxidation and

pattern of substitution of the B ring, generate different classes of flavonoids, while individual compounds within a class differ in the pattern of substitution of the A and C rings.[7]

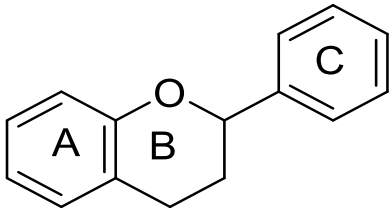

Figure 5: Basic structure of flavan. Aron, P. and Kennedy, J. (2008).[15]

The principal types of flavonoids, shown in **Figure 6** are: flavones (common in grains and aromatic herbs), flavanones (found in legume tissues), isoflavones (most often found in legumes and some beans like soybeans), flavonols (predominant in the skin of vegetables and fruits like quercetin), flavanonols (found in legume seeds), flavan-3-ols (found in tea leaves), and anthocyanidin (act as visual signals for pollinating insects).[7,15] The activity of flavonoids as antioxidants depends on the redox properties of their hydroxyphenolic groups and the structural relationship between the different parts of the chemical structure.[7]

According to Zhu and Gao[16], when flavonoid extracts are concentrated in nanocapsules, they are protected until they reach the active site of melanin synthesis, where they exert a powerful reducing action and antiradical activity, acting as a substrate competitor for tyrosinase (the enzyme that catalyzes the oxidation of phenols).[16]

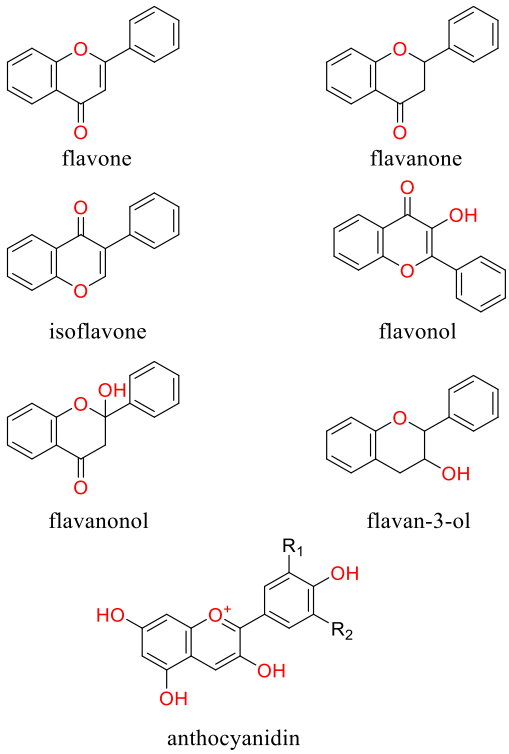

Figure 6: Different types of flavonoids. Pietta, P. (2000).[7]

Anthocyanidins and their glycosidic counterparts like anthocyanins are natural pigments responsible for the red and bluish-red colors of vegetables and fruits, being abundant in berries and red grape. Thereby, the interest of anthocyanins pigments has recently intensified due to their pharmacological, therapeutic and antioxidant properties.[17]

1.3.1. Anthocyanins

Anthocyanins, part of the family of flavonoids – anthocyanidin, are water-soluble pigments. They are responsible of red, purple or blue color of fruits and due to this property, it has been recognized as a food colorant. Within the main components of anthocyanins are malvidin and delphinidin that constitute almost 75% of all identified anthocyanins.[14] In general, anthocyanins have: delphinidin (27%–40%), malvidin (22%–33%), petunidin (19%–26%), cyanidin (6%–14%) and peonidin (1%–5%).[14]

The general structure of an acylated anthocyanin consists of an anthocyanidin structure bonded to a glycoside and acyl group, represented in **Figure 7**. However, under sudden temperature changes, the glycosylated and acylated compounds can be degraded, hydrolyzing the sugar and leaving the anthocyanidin structure.[6]

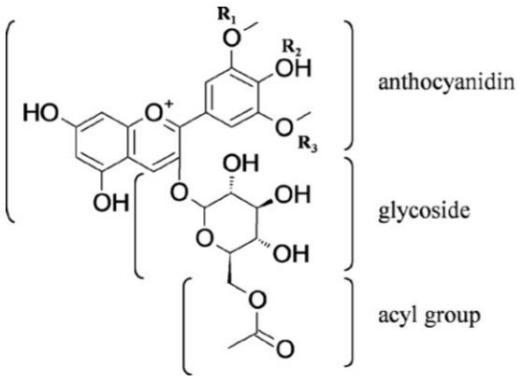

Figure 7: Skeletal structure of an acylated anthocyanin. Barnes et al. (2009).[6]

The substitution of R1, R2, and R3 carbon group indicated in **Figure 7** by the different groups given in **Table *2*,** define the type of basic anthocyanidin. The general structure of anthocyanin depends on the type of glucoside and the acyl group bonded to the anthocyanidin structure. For example, if the anthocyanin of interest is cyanidin, its structure is conformed by an anthocyanidin structure with galactose as glycoside group, so in this case, there is not an acylation process in the cyanidin structure.

Table 2: Anthocyanidin substituents found in natural anthocyanins. Barnes et al. (2009).[6]

Anthocyanidin	R1	R2	R3	Glycoside	Acylation
Delphinidin	OH	OH	OH		
Cyanidin	OH	OH	H	Galactose	
Petunidin	OMe	OH	OH	Sambubiose	Acetoyl , Malonyl
Pelargonidin	H	OH	H	Glucose; arabinose	Coumaroyl
Peonidin	Ome	OH	H	Rutinose; xylose	
Malvidin	OMe	OH	OMe		

Anthocyanins are polar compounds[18] that can be dissolved in different polar solvents such as water, alcohols (the most used is methanol[6]) and acetone. Also, they are pH dependent[18], so the color associated with the fruit is not always the same, the color varies in red, purple or blue. Due to their therapeutic effects, anthocyanin pigments are commonly used in medicines, with an average intake of approximately 180–215 mg/day, which is higher than that for other flavonoids.[19]

The antioxidant property of anthocyanins has been of interest and several methods of extraction have been proposed. Usually, the extraction solvents are acidified polar organic solvents, in which the amount of the acid to be used must be less than 7% of the total quantity;[6] strong acids such as trifluoroacetic acid, hydrochloric acid or weaker acids such as formic acid, acetic acid can be employed.[6] After extraction, follow of an appropriate purification and characterization processes developed by spectroscopic and chromatographic techniques, such as UV-Vis and Infrared (IR) spectroscopy, High Performance Liquid Chromatography (HPLC), Nuclear Magnetic Resonance (NMR) and Mass spectrometry (MS). The qualitative and quantitative analysis of anthocyanins has become extensive work but is still limited by the available calibration standard.[1,6]

1.4. Emulsions

An emulsion is a dispersion of an immiscible liquid into another; the dispersed phase is in the form of droplets which are dispersed in the surrounding liquid, also called the continuous phase (**Figure 8**). Emulsions, considered a type of colloids, are thermodynamically unstable systems because their two phases are immiscible. To add stability to the emulsion, a surfactant is needed.[20]

For systems in which the refractive index of dispersion medium differs from the dispersed phase, the color of the emulsion is opaque,[1] for which the dispersed-phase particle diameter of an emulsion is usually greater than 0.1 μm. In the case when the size particle falls to below

0.05 μm, the particles themselves are too small to produce any light interaction, and the emulsion becomes transparent.[21] Although, the emulsions can become transparent if the refractive index of both phases is the same, regardless of the diameter.[1]

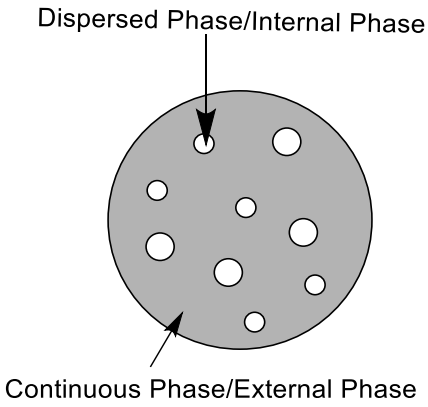

Figure 8: Structure of an emulsion.

The motion exhibited by colloidal particles in an emulsion is assumed to be a *Brownian motion*. In that case, individual particles continuously change direction as a result of their ceaseless random collisions with the dispersion medium, with other particles and with container walls.[22] The movement occurs regardless of the size of the particles, however, the size is inversely proportional to the fluctuations; fluctuations continuously occur at the molecular level when a liquid is homogeneous.[23] So, the smaller the particles, the more evident is their Brownian motion.

Emulsions are important in the production of medicine, cosmetic and food industry. Particularly, in the cosmetic field, many skincare products are based on emulsions,[1] and many efforts are spent turning the cosmetic industry into a very profitable industry.

1.4.1. Surfactants

Surfactants, also known as emulsifying agents, are compounds that help emulsions to make their phases miscible with each other.[22,23] They have a hydrophobic tail (or non- polar side) and a hydrophilic head (or polar side). In **Figure 9A** is shown a representation of a surfactant. When there is a critical amount of emulsifiers, their molecules self-associate forming micelles where the polar head comes into contact with water and oil with the non-polar tail as shown in **Figure 9B**.

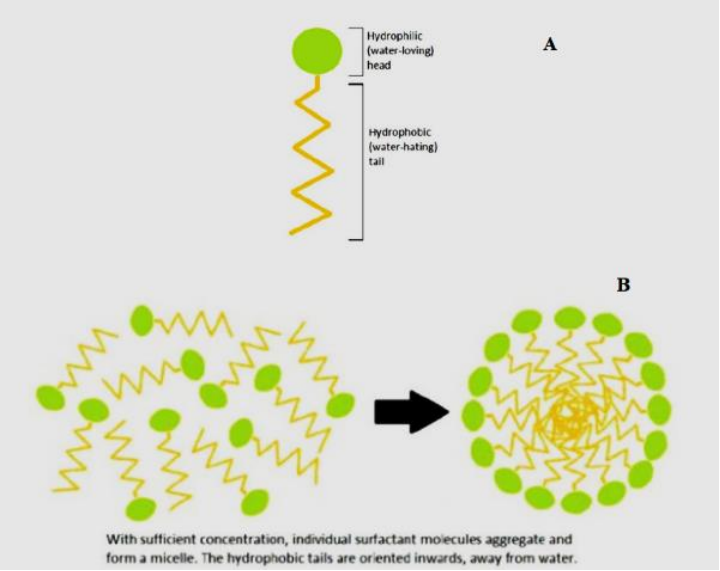

Figure 9: Representation of (A) a surfactant molecules and (B) Self-assembly of surfactants forming micelles. Shapiro, J. (2018).[24]

Surfactants act as detergent because the micelles formed help to remove dirt. This process consists in that hydrophobic tails of surfactant molecules surround the dirt as they are attracted to it, while hydrophilic heads trap dirt-forming micelles with dirt in the center of the structure. A scheme of this mechanism is shown in **Figure 10**. Apart from detergent and stabilizer properties, surfactants have foaming, wetting, and thickness properties.

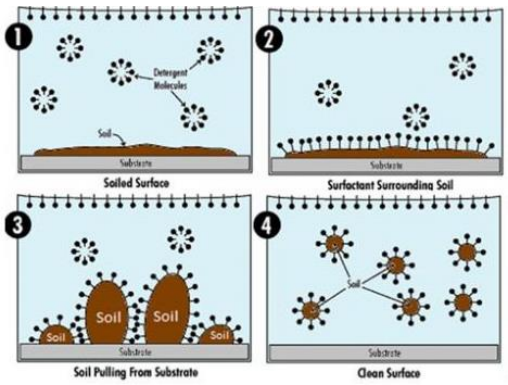

Figure 10: Representation of dirt removal process. 1) Soiled surface, 2) Surfactant surrounding soil, 3) Soil pulling from substrate, 4) Clean surface. Shapiro, J. (2018).[24]

Surfactants can be classified depending on their solubility, such as hydrophilic (soluble in water) or hydrophobic (soluble in lipids or fats), and it depends on the Hydrophilic-Lipophilic Balance (HLB).[25] Due to hydrophilic head is electrically charged, surfactants also can be classified depending on the sign (or presence) of this charge. Thus, there are ionic (anionic and cationic), non-ionic, and amphoteric surfactants.

Anionic surfactant: It has the hydrophilic group with a negative charge. The hydrophilic side dissociates into anions in aqueous solutions.[24,25] Negative charge in the hydrophilic end, helps the surfactant molecules to catch the dirty particles and that property is useful in the industry of cleansing products.[25] They are hydrophilic surfactants and create a lot of foam when mixed among the components of the products in which they have been implemented.[24] The most common hydrophilic groups of anionic surfactants are: carboxylate ($–COO^-$), sulfate ($–OSO_3^-$), sulfonate (SO_3^-), carboxybetaine ($–NR_2$- CH_2COO^-), and sulfobetaine ($–N(CH_3)_2C_3H_6SO_3^-$).[25]

Cationic surfactant: It dissociates into cations. They have a positive charge in the hydrophilic head and they are hydrophilic surfactants. They are used in the production of anti-static products and disinfectants due to their charge.[24] One of the groups usually associated with cationic surfactant is quaternary ammonium ($–R_4N+$).[25]

Non-ionic surfactant: It does not have a charge on their hydrophilic end. This type of surfactants is very useful as emulsifying agents. Some nonionic surfactant can be non-foaming or low-foaming.[24] Due to non-ionic surfactants are considered as low foam cleaners, their use avoid separation of the phases in the emulsion. Also, they can be hydrophilic or lipophobic surfactants. The most common group of non-ionic surfactant are glycerol and sorbitol groups.[25]

Amphoteric surfactant: It dissociates into anions and cations in a solution. The dissociation depends on pH and the positive and negative charges cancel between them creating a net charge of zero (zwitterionic).[24] This type of surfactant can acts as ionic surfactant in alkaline solutions and as cationic surfactant in acidic solutions.[24]

1.4.2. Types of emulsions

1.4.2.1. Oil in water emulsion (O/W)

In O/W emulsion, the water phase is the continuous phase in which the micelles are formed, and the dispersed phase is the oil phase. In this type of emulsions, the orientation of surfactants is the hydrophilic group on the outside of the colloidal particle and the lipophilic group on the inside.[1]

1.4.2.2. Water in oil emulsion (W/O)

In W/O emulsion, the oil phase is the continuous phase and the dispersed phase is the water phase. In this type of emulsions, the orientation of surfactants is the hydrophilic group on inside and the lipophilic group on outside of the colloidal particle.[1]

Both emulsions can be identified by electrical conductivity measurements, dilution test and by the dye method. O/W emulsions have higher electrical conductivity values than W/O emulsions.[1,22] Generally, W/O feels oily and O/W feels creamy texture; in both cases, its appearance may be colored by the dyes which solubility in one of the phases identifies the type of emulsion.[22] Also, there are double o multiple emulsions denoted as W/O/W and O/W/O. These types of emulsions can be seen by a microscope, and the three phases involved can be detected,[1] that is the innermost phase, the intermediate phase and the outermost phase as is shown in **Figure 11**.

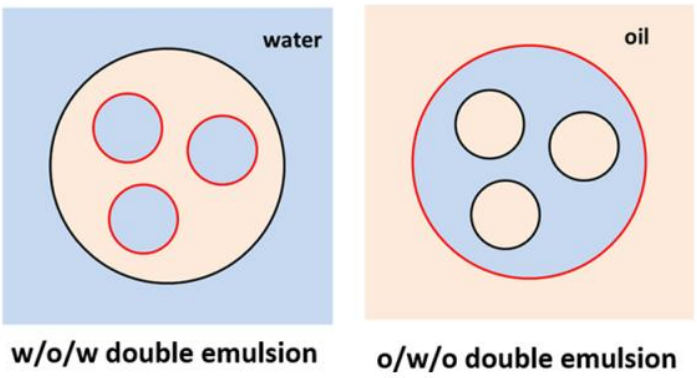

Figure 11: Representation of W/O/W and O/W/O double emulsion. Thompson, K. et al. (2015).[26]

1.4.3. Hydrophilic/Lipophilic Balance

The type of emulsions depends on the balance between hydrophilic and lipophilic properties of the emulsifier.[22] The HLB is a relative measure of the contribution of each region of the molecule. Usually, it is measured on the arbitrary scale of 0-20 known as Griffin scale, in which a higher value of HLB corresponds to a hydrophilic surfactant and a lower value of HLB corresponds to a lipophilic surfactant as depicted in **Figure 12**.[22] HLB is used as an indicator to choose a surfactant for a specific application.

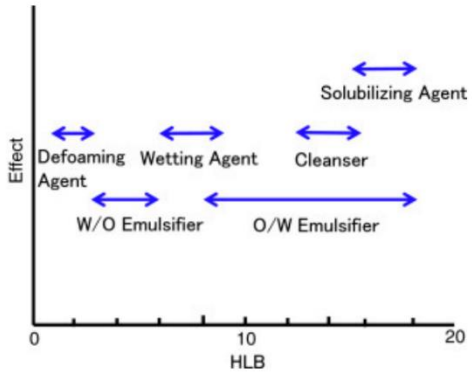

Figure 12: Index of choosing a surfactant. Nakama, Y. (2017).[27]

For example, lecithin and cetyl trimethyl ammonium bromide (CTAB) has HLB values equal to 4.5 and 10, respectively, having a lipophilic behavior. [28,29] HLB number can be calculated according to the nature and concentration of the used emulsifier. To stand out, HLB changes with the temperature change, when temperature increases, the hydration of lyophilic groups decreases and the surfactant becomes less hydrophilic giving a lower HLB. So, it means a great disadvantage since HLB makes allowance for temperature effects.[23,30]

1.5. Separation and characterization techniques for skincare cosmetics

In recent years, skincare products are analyzed from their raw material components and as a final product to provide a complete characterization of cosmetics.[1] In the cosmetics, the analysis is carried out in three stages: **1)** analysis required to confirm that the raw materials for the preparation of the products are of high quality, **2)** analysis to guarantee the amount used of their ingredients and **3)** analysis to ensure that products obey the highest quality standards having good manufacturing practices.[1] Because of this, a good selection of appropriate analysis techniques is important.

1.5.1. Separation techniques

Separation techniques are most commonly used because the final cosmetic product and their raw materials are often mixtures of several ingredients.[1] Among the separation techniques usually used are: distillation, evaporation, filtration, fractional precipitation, liquid-liquid extraction, Chromatographic techniques, and ashing.

Distillation and evaporation are techniques used to remove solvent when its boiling point allows.[1]

Filtration after evaporation is used to separate substances soluble in solvents from those insoluble in them.[1]

Fractional precipitation used by adding poor solvents to good solvents such as butanol, n-heptane.[31] For example, separation of nylon from metacresol solution of nylon.[1]

Liquid-Liquid extraction for separate two immiscible extraction.[1]

Ashing for separate inorganic solvents by decomposition at high temperature.[1]

Chromatographic techniques

Column Chromatography, it is essential for the analysis of cosmetics. The most common is the Liquid-Solid Chromatography which consists in a stationary phase (like silica gel and alumina) and a mobile phase.[1] As stated in the book *New Cosmetic Science* "there is no device for continuously monitoring the eluted components, in order to widen its scope of use, it is necessary to employ a standard mobile phase system, such as stepwise elution, and to analyze the eluted components by some other means because the separation varies with the activity of the adsorbent and the amount of solvent used."[1]

Based on the principle of Thin Layer Chromatography (TLC), for the separation of compounds, the mobile phase should have different properties from the stationary phase, which means, the mobile phase must not have the same polarity as the stationary phase.[32] The compounds in the mixture, under the influence of the mobile phase, travel over the surface of the stationary phase, and during this movement, the compounds with higher affinity to stationary phase travel slow while the others travel faster.[32,33]

Gas chromatography (GC) is a technique that can provide qualitative information by indicating retention times.[1,34] Because it provides qualitative information, it can be used to identify raw materials in cosmetics, oil, fats, and waxes. GC can analyze compounds of long carbon numbers, around 50-60 carbons.[1] GC uses several types of detectors such as thermal conductivity, ionization[35] which are quite sensitive for the detection of most chemical compounds. There are also flame photometric detectors (to selectively detect nitrogen, phosphorus, and sulfur) and electron capture detectors (to know the retention time of the compounds).[1]

High Performance Liquid Chromatography (HPLC) analyze more compound than Gas Chromatography. HPLC is a technique used to separate the components of a mixture based on different types of chemical interactions between the analyzed substances and the chromatographic column. It is a technique more quantitative than qualitative. The most commonly used detection device in HPLC is the ultraviolet detector for quantitative analysis since it is considered selective because it saves time by adjusting to the wavelength of the substance that needs to be analyzed, which simplifies the cleaning operation of sample.[1]

Gel permeation chromatography (GPC) is a quantitative analysis based on molecular weight. In the cosmetics industry, it is widely used since the polymers of the products can be detected by their molecular weight, size, and structure.[1] Moreover, in other industries such as the

pharmaceutical and food industry, with GPC it is possible to establish parameters to improve the product performance.[1]

1.5.2. Characterization Techniques

Structural analysis by X-Ray diffraction

X-ray diffractometry is a technique for the characterization of compounds using X-rays. The separation of titanium dioxide, talc, kaolin, and iron oxides frequently used in cosmetics is extremely difficult without changing their forms and states, for which qualitative analysis is carried out based on the patterns of diffraction peak.[1] The diffraction peaks vary according to the sample matrix and how the sample is packaged in the cell for analysis. For cosmetics, it is difficult to reflect with X-rays, the state of a cosmetic sample due to its high content of inorganic raw materials such as iron, aluminum, magnesium, silica, titanium, etc.[1] On the other hand, quantitative analysis in X-rays can also be employed but present limitations.[1]

Chemical Characterization techniques

Infrared Spectroscopy (IR) is a technique that provides information based on functional groups.[1,36,37] Vibrational infrared region is from 4000 cm^{-1} to 400 cm^{-1} which provides structural information about a molecule.[37] Infrared radiation is a quantized process and in cosmetics, it is used for daily quality control for testing of raw material[1,37] and to confirm the presence of functional groups of a specific molecule.

Nuclear Magnetic Resonance (NMR) is an analysis to determine the structure of substances.[37] The analysis can be based on carbon type (^{13}C-NMR) or proton (^{1}H-NMR) types or both. Both techniques can be used to evaluate the surfaces of inorganic powders used as cosmetics material that has been treated with silicone, as well as in the analysis of the states of emulsions with liposomes when used together with exchange reagents.[1]

Mass Spectroscopy (MS) is also a structural analysis due to the molecular weight data it provides. Molecular weight data are used to identify unknown compounds and to quantify known compounds. For cosmetics, it is usually used with gas chromatography for the best results.[1] The mass spectrometer bombards the sample with a high-speed electron current where the substance loses electrons and fragments into different ions, radicals, and neutral molecules. Subsequently, the ions are collected and distributed in the spectrum according to their mass ratio/charge (m/z).[37]

Atomic Emission Spectroscopy (AES) and Atomic Absorption Spectroscopy (AAS) are used as micro quantitative analysis in cosmetics for a rapid determination for lead, arsenic and other metals.[1] Besides, Atomic Emission Spectroscopy can also be used as qualitative analysis for inorganic compounds without the need to separate them, used for the preparation of cosmetics.[1] Then, for AES, samples do not need pre-analysis treatment.

Ultraviolet Visible Spectroscopy (UV-Vis) is a technique that analyzes molecules based on transitions between electronic energy levels where wavelengths range from 190 nm to 800 nm.[37] As a molecule absorbs energy, an electron pass from highest occupied molecular orbital (HOMO) to a lower unoccupied molecular orbital (LUMO). Theoretically, the energy difference between HOMO and LUMO varies from 125 to 650 kJ/mol.[37] In all compounds other than alkanes, the electrons may undergo several possible transitions of different energies.[37] Some of the most important transitions are illustrated in **Figure 13**.

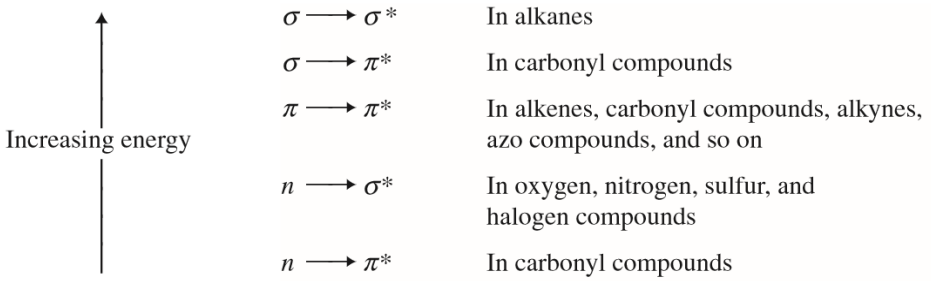

Figure 13: Most important transitions in Ultraviolet Visible Spectroscopy. Pavia, D. et al. (2013).[37]

The main advantage of this type of spectroscopy is to develop high standards of Quality Control and production processes to improve the shelf life of products in different industries such as food, cosmetics, and pharmaceutical.[38]

2. CHAPTER 2. JUSTIFICATION – OBJECTIVES

2.1. PROBLEM STATEMENT

At present, due to several environmental factors and health problems, the skin is usually affected mainly in the epidermis, in particular, the outermost layer, called stratum corneum showing the first signs of damage as dehydration. Dehydrated skin has a rough appearance and sometimes presents desquamation (xerosis).[20] For help with this problem, moisturizers are one of the most important classes of cosmetic products due to preventive action against xerosis and the delay of premature aging. To improve aspects of their skin, people use skincare cosmetics, which in some cases are based on emulsions conveniently prepared, exploiting the properties of each one of their components.

Many cosmetics have been associated with a large list of sophisticated reagents with particular functions, for improving their texture and fluidity, and overall, for increasing their chemical and physical stability. Also, it is possible to prepare cosmetics using natural components from fruits like blueberries, in particular moisturizers and antioxidants creams, from readily available reagents, provided that the functions of each of the components used are known. The analysis of the functionalities of these components allows proposing a simple protocol for the preparation of creams that could serve as the basis for more sophisticated future proposals.

In this sense, as a **Hypothesis**, the use of anthocyanins, rich in vitamins A and C, in the formulation of W/O/W emulsion, would lead to generate a product with antioxidant and moisturizing properties, as well as with the capacity of protecting the skin from ultraviolet (UV) radiation.

2.2. GENERAL AND SPECIFIC OBJETIVES

- *General objective*

Encapsulate Blueberries Extract in a double emulsion (W/O/W), based on a natural compound with antioxidant and moisturizing properties.

- *Specific objectives*
  - Extraction and Characterization of anthocyanins from Blueberries Extract.
  - Evaluation of the antioxidant activity of the extract.
  - Preparation and characterization of W/O and W/O/W emulsions.
  - Evaluation of the stability of emulsions prepared.
  - Estimation of UVA and UVB absorption of double W/O/W emulsion prepared

3. CHAPTER 3. METHODOLOGY

3.1. Reagents – Materials – Equipment

The chemical reagents used in the different stages of the project are listed in **Table 16** in **Annex 2** as well as raw materials, some of them being natural products, acquired in the local stores. It is also indicated the brand of the different reagents and materials mentioned, except for lignin, which was extracted in the Laboratory of Chemistry at Yachay Tech University. The physical-chemistry properties and the information about the level of risk that a substance can represent for human safety and health are given in **Annex 1**, in **Table 15**.

Also in **Annex 2**, there are indicated the laboratory materials and equipment required for the extraction of natural sources, preparation of emulsion and characterization processes, as well as for the respective assays for the evaluation of the antioxidant and moisturizing capacity of the obtained products.

3.2. Drying procedure of Blueberries fruit

It is known that the most used method for drying fruits is by using an oven to reduce humidity content, preserving them and extending their shelf life.[39] Drying of blueberries was carried out in the laboratory in the Drying Stove POL-EKO APARATURA. This procedure is necessary to eliminate the water content of blueberries. The procedure followed is indicated below:[40]

- The dryer was turned on to reach a stable temperature.
- Different samples of blueberries denoted as A and B were separated, weighed and identified, previously washed. Sample A: 123.78 g and Sample B: 126.07 g.
- By a multistage drying, the temperature profile was in the range 50-90 °C for 3 days.
- In order to calculate the loss of water content, the relative humidity (Rh) is determined by $Rh\ (\%) = \frac{Initial\ Weight - Final\ Weight}{Initial\ Weight} \times 100$, named as Equation 1.
- Both samples were joined and ground with a Coffee Grinder DAEWOO, for further extraction. The joined and ground samples are denoted as E*lement 1* of both samples. This fraction was stored at 12 °C.

3.3. Obtaining of Blueberries fruit extract

For this procedure, Element 1 was used. The steps listed below summarize the procedure followed:[40]

- 10 g of a sample of Element 1 was mixed with 75 mL of extracting solvent consisting of methanol: water: acetic acid (0.2 M) at a ratio of 37.5:36:1.5.[40]

- The extract was centrifuged in a Centrifuge Thermo Fisher Scientific SORWALL Legend XTR at 13000 rpm for 20 min, under the condition of 15 °C.

- After removing the supernatant, the residue (called *Residue 1*), was mixed again with 75 mL of the extracting solvent followed by centrifugation at the same condition above.

- Both supernatants were collected, combined and evaporated using a rotary evaporator Buchi – R210 at 35 °C.[40]

- The residue obtained after evaporation with viscous appearance, called *Residue 2*, was mixed with 5mL of a solution of formic acid:water (3:97). This mixture was named as *Crude Extract*.

- A TLC analysis of the Crude Extract was done using a mixture of formic acid:methanol with a ratio of 3:97 as the mobile phase.

- To reach the separation of the anthocyanins and other phenolic compounds, a chromatography column was loaded using 100 mL of formic acid:methanol (3:97) solution as eluent. The representation of this process is illustrated in **Figure 14**.

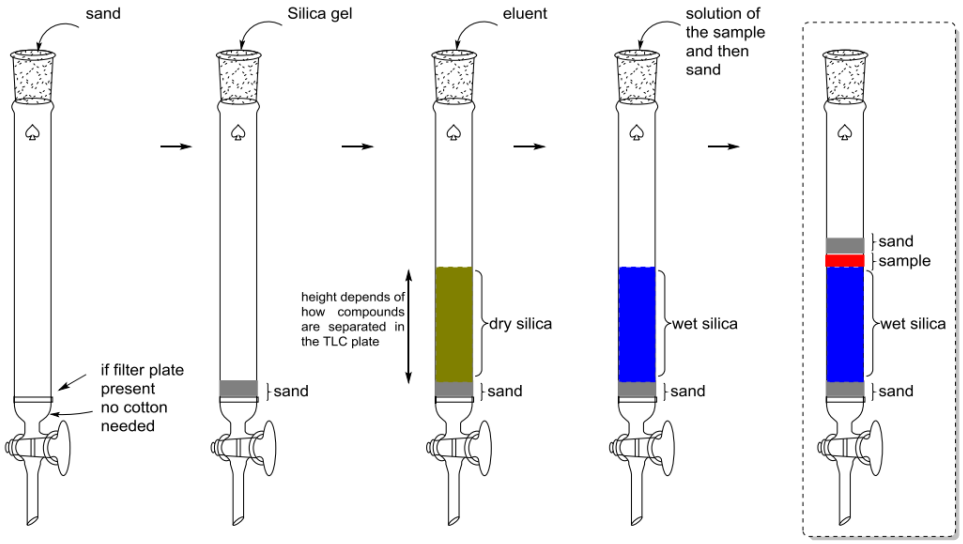

Figure 14: Representation of the Chromatography Column process. López and Makowski (2018).[41]

- Then, there were obtained two fractions. *Fraction 1* was the first fraction and it was colorless. *Fraction 2* was the colored fraction of interest and was evaporated using the rotary evaporator Buchi – R210 at 35 ºC until a residue was obtained, termed as *Residue 3*.

- Residue 3 was viscous, was weighed and then was dissolved with 3 mL of a mixture of formic acid:methanol:water (3:14.55:82.45) called as *Blueberries Extract* and as *Anthocyanin Extract*.

- The pH and density were measured.

- The Blueberries Extract was identified using UV-Vis and IR spectroscopy which was compared to what has been reported in the literature.

3.4. Evaluation of Antioxidant Capacity

3.4.1. Determination of total phenolic content of Blueberries Extract

Total phenolic contents were determined by spectroscopy techniques using Folin-Ciocalteau reagent.[40,42] The associated procedure consisted of:

- 20 μL of Blueberries Extract solution was mixed with 1.16 mL of distilled water, 100 μL of Folin-Ciocalteau reagent (10%), and with 300 μL of sodium carbonate solution (Na_2CO_3) (20%).

- The final solution was incubated for 30 minutes at 40°C, and then the absorbance of the solution was measured at 760 nm using UV-Vis Spectrometer Perkin Elmer/Lamba 1050.

- For this test, gallic acid was used as a reference standard. By previous analysis developed in the Laboratory of Chemistry at Yachay Tech University, the calibration curve was constructed for this standard.

- Gallic acid was used as a reference standard.

- Total phenolic content was calculated using the Equation 2 established by Genwali et al.[43,44]

$$\textit{Equation 2: } Total\ phenolic\ content\ \left(\frac{mg\ GAE}{g\ EDW}\right) = \frac{GA \times V \times DF}{m}$$

where *GA* is the concentration of gallic acid in $\frac{mg}{mL}$; *V* is the volume of the extract in mL, *DF* is the dilution factor and *m* is the mass of the extract in gram.

- Finally, the results were expressed in terms of Estimated Dry Weight of Blueberries Extract (EDW) and Gallic Acid Equivalent (GAE) by the units: $\frac{\mu g\ GAE}{mg\ EDW}$ [40,42-44] using the Equation 2.

3.4.2. Determination of total flavonoid content

This analysis uses the aluminum chloride colorimetric assay. The procedure was established by Meryem et al.,[42] in which 0.5 mL of blueberry extract was mixed with 0.5 mL of 2% $AlCl_3$.[42] Then, the solution was incubated for 1 hour at room temperature and the absorbance was measured at 420 nm and 700 nm. Quercetin was used as a reference standard.

For calculation of total flavonoid content, it was used the Equation 3[44] where *QE* is the concentration of quercetin equivalent in $\frac{mg}{L}$, *V* is the volume of the extract in a liter, *DF* is the dilution factor and *m* is the mass of the extract in grams. Total flavonoid content was expressed as microgram Quercetin Equivalent (QE) and Estimated Dry Weight of Blueberries Extract (EDW), $\frac{\mu g\ QE}{mg\ EDW}$.[42]

$$Equation\ 3:\ Total\ flavonoid\ content\ (\frac{mg}{g}\frac{QE}{EDW}) = \frac{QE \times V \times DF}{m}$$

3.4.3. Determination of total anthocyanins

According to Somsong et al.[40], total anthocyanins determination was performed using spectroscopic pH-differential methods established by Giusti and Wrolstad.[40] Samples were diluted in two buffers, one of potassium chloride (pH 1.0) and the other of sodium acetate (pH 4.5) and then measured at 520 and 700nm using UV-Spectrometer Perkin Elmer / Lamba 1050.[40] Buffers were prepared as described below:

- *Potassium chloride buffer pH 1.0:* 0.0759 g of potassium chloride KCl was mixed with 40 mL of distilled water and 22 drops of concentrated HCl. The solution was placed in a 50 mLvolumetric flask.

- *Sodium acetate buffer pH 4.5:* 2.2978 g of sodium acetate CH_3COONa was mixed with 40 mL of distilled water and 30 drops of concentrated HCl. The solution was placed in a 50 mL volumetric flask.

In order to measure the pH value, it was used a pH meter Mettler Toledo. Finally, for the analysis in the UV Spectrometer, 1 μL of the Anthocyanin Extract was mixed in 50 mL of each buffer.

It was proposed a quantitative relationship based on the absorbance difference recorded for the extract under the different acid pH conditions between wavelengths 520 and 700 nm,[45] as

represented in Equation 4. $A_{520\,nm}$ is the absorbance value at 520 nm and $A_{700\,nm}$ is the absorbance value at 700 nm.

$$Equation\ 4:\ A = (A_{520\,nm} - A_{700\,nm})_{pH\ 1.0} - (A_{520\,nm} - A_{700\,nm})_{pH\ 4.5}$$

Then, the concentration in mg/L of anthocyanins in the extract was determined by the Equation 5, where the molecular weight of cyanidin-3-glucoside is 449.2 g/mol, A is the absorbance value at different acid pH conditions, ε is the molar absorptivity coefficient equal to $26900\,\frac{L}{mol \times cm}$, L is the path length equal to 1 cm and *DF* is the dilution factor.

$$Equation\ 5:\ C_{extract} = \frac{A}{\varepsilon \times L} \times Molecular\ Weight \times \frac{10^3 mg}{g} \times DF$$

3.4.4. Reducing power determination of Anthocyanin Extract

The ferric reducing capacity of extracts was investigated by using the potassium ferricyanide-ferric chloride method.[42,46] For this determination, the procedure indicated below was followed:[42,47]

- 0.2 mL of each of Blueberries Extract at different concentrations: 10%, 15%, 20%, 25%, 30%, were mixed with 2.5 mL of phosphate buffer (0.2M, pH 7.2), and with 2.5 mL of potassium ferricyanide $K_3Fe(CN)_6$ (1%).

- The resulting solutions were incubated at 50°C for 20 minutes. The reaction was stopped by adding 2.5 mL of 10% (v/v) trichloroacetic acid.

- Solutions were centrifuged at 1000 rpm for 10 minutes.

- 2.5 mL of the supernatant solution was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3 \cdot 6H_2O$ (0.17%). The absorbance of the resulting solution was measured at 700 nm.

- The same procedure was followed using ascorbic acid. The reducing power of ascorbic acid was also determined for comparison.

3.4.5. Hydrogen peroxide scavenging activity

3mL of a solution of hydrogen peroxide (40 mM) was mixed with five drops of phosphate buffer (pH 7.2), this was called standard solution.[48] 2.2 mL of standard solution was mixed with 0.8 mL of Anthocyanin Extract 20%. The absorbance of the sample at 560 nm was determined after 10 minutes against the standard solution.[48,49] At the same wavelength, also the absorbance of the extract was measured in order to be used as control.

According to the literature, the recommended equation for the evaluation of scavenging activity of Blueberries Extract was:[48,49]

$$Equation\ 6:\ (H_2O_2\ scavenging\ activity)\% = \frac{A^o - A}{A^o} \times 100$$

where A^o is the absorbance of the control and A is the absorbance of the standard solution with extract.

3.4.6. Phosphomolybdenum assay

The main consideration for phosphomolybdenum assay was described by Sharadanand Phatak and Subhash Hendre[50] stablishing that the P-M assay evaluates, by incubation at high temperature, the reduction reaction between the antioxidant, the oxidant and the molybdenum ligand. Phosphomolybdenum assay (P-M assay) is a method used to measure the antioxidant activity. It consists on the reduction of molybdenum from oxidation state from +6 to +5, and of the reduction reaction is confirmed by the formation of a bluish-green colored phosphomolybdenum (V) complex at acid pH.[50,51]

Phosphomolybdenum assay was used to estimate the total antioxidant activity of Anthocyanin Extract, for which 1 mL of molybdate reagent solution was mixed with 0.1 mL of Anthocyanin Extract.[50] Molybdate reagent solution consists of 1ml of 0.6 M sulfuric acid, 1 mL of 28 mM sodium phosphate and 1 mL of 4 mM ammonium molybdate were made up volume to 50 mL by adding distilled water.[50] Ascorbic acid is used for comparison. Then, the absorbance was measured at 695 nm.

3.5. Preparation of primary emulsion

Anthocyanin Extract was incorporated in the water phase for its better distribution, for which there is enough polarity affinity between water and extract.

3.5.1. Preparation of primary emulsion

The samples of emulsions were prepared with different concentrations of surfactant in order to form the emulsion with the desired texture similar to a cosmetic cream. The most convenient emulsion samples had a ratio aqueous phase:oil phase of 30:70. The aqueous phase was composed of water, Anthocyanin Extract and cetyl trimethyl ammonium bromide (CTAB, cationic surfactant[52]). The oil phase was composed of coconut oil and lecithin (amphoteric surfactant[53]). The composition of emulsion tested is given in **Table 3**.

Table 3: Composition of primary emulsions.

N°. Emulsion	Oil Phase	Water Phase
I	22 g of coconut oil Karay	100 μL of Anthocyanin Extract 8 mL water 1 g CTAB
II	22 g of coconut oil Weir	100 μL of Anthocyanin Extract 8 mL water 1 g CTAB 0.12 g NaCl
III	22 g of coconut oil Weir	100 μL of Anthocyanin Extract 8 mL water 1 g CTAB
IV	22 g of coconut oil Weir	100 μL of Anthocyanin Extract 8 mL water 1 g CTAB 0.24 g NaCl
V	21.56 g coconut oil Coco-Freeze 20 μL Lecithin	87.62 μL of Anthocyanin Extract 7.96 mL water 0.966g CTAB
VI	21.45 g coconut oil Coco-Freeze 25 μL Lecithin	438.12 μL of Anthocyanin Extract 8.14 mL water 0.45 g CTAB

Also, another ratio aqueous phase:oil phase, 60:40, was used to prepare emulsions maintaining the basic composition of aqueous and oil phase, and adding other compounds in the phases to evaluate if the type of emulsion can be reversed. The composition of the emulsion is described in **Table 4**.

Table 4: Composition of primary emulsion.

N°. Emulsion	Oil Phase	Water
VII	18.07 g of coconut oil Karay	- 100 μL of Anthocyanin Extract 25mL water 1 g glycerin 1 g CTAB
VIII	18 g of coconut oil Karay 0.45 g Lecithin	167 μL of Anthocyanin Extract 25 mL water 1.07 g glycerin 0.8 g CTAB

For a better mixing of components, both phases had to be heated separately, the aqueous for 10 minutes and the oil phase for 20 minutes. Once the two phases were well mixed, they were heated for 30 minutes to achieve homogeneity in the primary emulsion. It was decided which

one of the prepared emulsions had the best composition for the preparation of more convenient W/O emulsion for finally achieve the desired double emulsion W/O/W.

3.5.2. Dilution test for determining the type of primary emulsion

Dilution test was applied to determine the type of the primary emulsion, either oil-in-water (O/W) or water-in-oil (W/O).[54] This is a quick method that depends on the miscibility with respect to the water or the oil.[54, 55] For this test, the water phase contained 1 mL of water and 62.5 μL of Anthocyanin Extract and oil phase contained 1 mL of coconut oil, each of them were poured into different beakers. A few drops of the prepared emulsions were splattered into two beakers and the miscibility of the drops can be observed. If drops of emulsion were miscible with water, the type of emulsion was O/W emulsion. If they were dispersed in the oil phase, then this was a W/O emulsion because easy dispersion could perform only in the continuous phase.[54,55]

3.6. Conductivity Test for determining the type of primary emulsion

Emulsifying properties were determined by measuring the electrical conductivity with portable digital conductivity meter APERA USA, EC60 Premium of emulsions prepared in advance.[56] The electrical conductivity of emulsion was measured using an electrode of platinum black. The conductivity meter was placed inside the emulsion until it was in contact with the electrode and the conductivity value appeared on the screen.

3.7. Preparation of W/O/W type double emulsion

To potentialize the activity of anthocyanins and polyphenols found in blueberries, encapsulation is an interesting method. These abilities make polyphenols interesting for anti-aging purposes in cosmetic formulations. With the encapsulation of emulsions, converting them in a double emulsion, it is expected to have a protection against oxidation and thermal degradation, thus contributing to increase the useful life of the encapsulated active ingredient.

Once the primary emulsion was formed, the preparation of double emulsion was carried out.[54] The primary type of W_1/O emulsion is mixed with the outer aqueous phase (W_2).[54] The outermost aqueous phase was composed of water and a very small amount of lignin, which can be considered an anionic surfactant[57]. To prepare $W_1/O/W_2$ emulsion, it was taken 10 % of the aqueous phase (W_1) and 90% of the oil phase (O) based on the sample conditions V and VI (**Table 3**), they were mixed and stirred for 10 minutes. Then, it was taken 20% of the W_1O emulsions based on the proportions described in **Table 5** and mixed with 80% of outer aqueous

phase W_2. Primary emulsion was added to the outer aqueous phase gradually for 2 minutes.[54] To help mix well, it was heated for 20 minutes at 40 °C and sonicated at 5 °C for 10 minutes.

Table 5: Conditions to prepare primary emulsions (W/O/) for the production of W/OW double emulsion.

N° sample	Water phase (W_1)	Oil Phase (O)
A	1% of Anthocyanin Extract Water CTAB	2% lecithin Coconut oil
B	5% of Anthocyanin Extract Water CTAB	2.5% lecithin Coconut oil

3.8. Optical Microscopy analysis of W/O, W/O/W emulsion and Anthocyanin Extract

To check if the emulsions were formed, all of the prepared systems were observed under an Optical Microscope LEICA and analyzed by the *LAS EZ software* program. The obtained emulsions were placed and displaced on the slide forming a very thin emulsion layer and covered with the coverslip. The images of the emulsions were taken at a magnification factor of 10X, 20X and 40X. The same procedure was performed for the extract to know the shape and size of the anthocyanin pigments.

3.9. Determination of UVA-UVB absorption of extract and emulsions

UVA-UVB Digital Meter GENERAL TOOLS UV513AB, 280-400 nm was used for this analysis. In a glass Petri dish, samples of previously prepared emulsions were spread evenly. The data thrown on the device's digital screen was immediately registered. The same procedure was also performed for Anthocyanin Extract.

3.10. Moisturizing determination of emulsions

This procedure involves placing a few amounts of the W/O/W double emulsions on a person's dehydrated skin to assess how long the emulsion can keep the skin hydrated. The moisturizing capacity of the prepared W/O/W double emulsion was evaluated by measurements using Digital Moisture Monitor for skin SK-IV for three hours at different intervals of time.

4. CHAPTER 4. RESULTS AND DISCUSSION

4.1. CHARACTERIZATION OF BLUEBERRIES EXTRACT

4.1.1. Analysis of dried blueberries

The Rh of blueberries fruits after being subjected to heat at different temperatures[40] in the range 50-90 °C was 36.93% for sample A and 37.76% for sample B. As can be seen in **Figure 15** and **Figure 16**, Sample B of dried blueberries had an appearance that loses much more color in relation to Sample A. According to literature, blueberry discoloration obtained does not affect the amount of anthocyanin pigments in the fruit.[40] However, the fruit has sensitive components to stereochemical changes related to C-ring carbons of the molecule to the formation of its isomer structure, so, at prolonged heat time, this could easily be observed in blueberries with excessive abrasion of the skin.[40,58,59]

Figure 15: Sample A of dried blueberries fruit

Figure 16: Sample B of dried blueberries fruit

Figure 17 shows the Anthocyanin Extract from dried blueberries which consisted of a viscous liquid of purple color with a pH ≅ 4 measure with pH measuring paper and 1.029 g/mL of density.

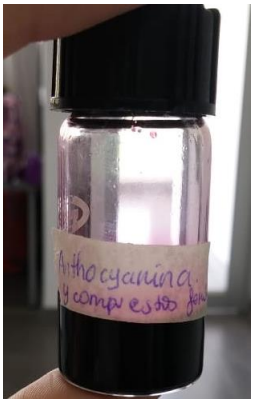

Figure 17: Anthocyanin Extract extracted in the laboratory at Yachay Tech University.

4.1.2. Thin Layer Chromatography analysis

TLC was performed before and after the chromatography column. Different solvents, such as formic acid, methanol, chloroform, ethanol, and hydrochloric acid were tested. However, due to the polar composition of anthocyanins and other phenolic compounds, and the silica gel available at the laboratory, a suitable mobile phase was not found for their identification.

In **Figure 18**, the TLC plate shows the crude extract composition before the chromatography column. Based on what is reported in the literature, anthocyanins vary from 0.55-0.72 using as mobile phase a mixture of hydrochloric acid:acetic acid:water (19:39.6:41.4).[36,60]

Figure 18: TLC plate of Crude Extract of anthocyanins in a mobile phase formic acid:methanol (3:97)

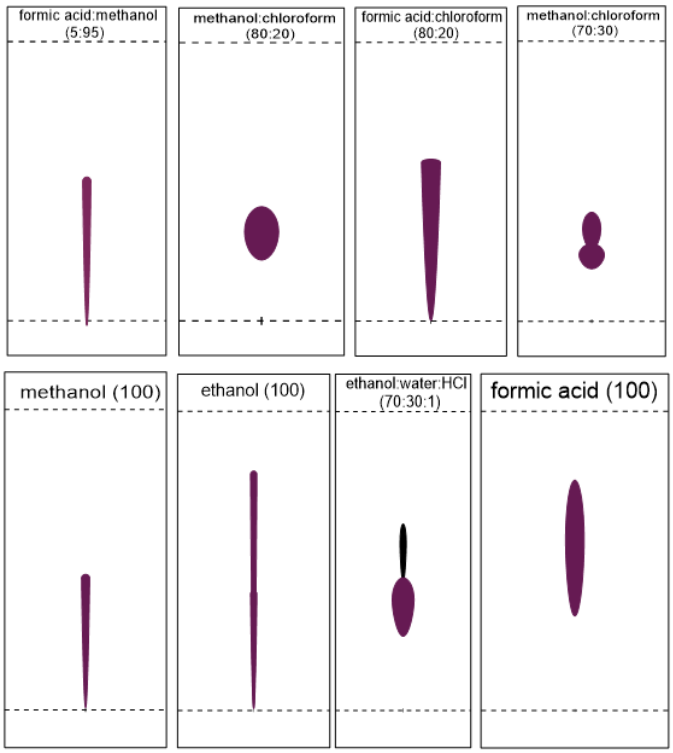

Figure 19: Different TLC plate analysis of Blueberries Extract at different concentration solvents.

Anthocyanin extract was analyzed by TLC with different solvents in different proportions. These tests, whose results are shown in the **Figure 19** were performed to evaluate any difference between TLC plates. It was not possible to report Rf values because the TLC could not be resolved with the right materials to compare the Anthocyanin Extract with other flavonoids like rutin and quercetin, and with sugars like lactose, fructose, glucose, starch, maltose, galactose, and saccharose.[36] For example, it could be appropriate the use of C18 reversed phase silica,[40] Sephadex LH-20 column chromatography,[61] chromatographic layer with hydroxyl groups,[36] or Kinetex PFP (150×2.1mm) core-shell column[62] which were not possible to acquire; in addition, to not having the appropriate solvents such as water:acetonitrile:formic acid (48.5:50:1.5)[61] or methanol:water:trifluoroacetic acid (70:30:1)[6].

With these results, unfortunately, an effective identification of the Crude Extract could not be achieved, but according to the different spots in TLC plates eluted, it is possible to infer that the extract would have anthocyanins and other phenolic compounds without ruling out the presence of sugars.[36] Thus, sugar moiety present in the extract increases the polarity of the anthocyanin molecule.[36]

4.1.3. Ultraviolet-Visible Spectroscopy results of Anthocyanin Extract for calculation of total anthocyanins

According to literature, energies associated with cyanidin molecules corresponds to -85339.24 kcal/mol as total energy, -3511.78 kcal/mol for binding energy and -17.97 kcal/mol for heat formation.[63] In the spectrum in **Figure 20**, the HOMO-LUMO transitions associated with the absorption bands were in accordance with the data reported[37], which are:

- At 280-290 nm it is associated to $n \rightarrow \pi^*$ transition due to a presence of a simple conjugated chromophore group in aromatic rings showing a prominent band around 280 nm.
- At 320 nm it is associated to $\pi \rightarrow \pi^*$ transition in phenolic compounds with $C_\alpha = C_\beta$ bonds conjugated with aromatic rings and $n \rightarrow \pi^*$ transition containing $C_\alpha = O$ groups.[64]
- At last, $\pi \rightarrow \pi^*$ (170-190 nm)[37] that must show the substitution group which is an auxochrome group that influences the wavelength shift in the range.

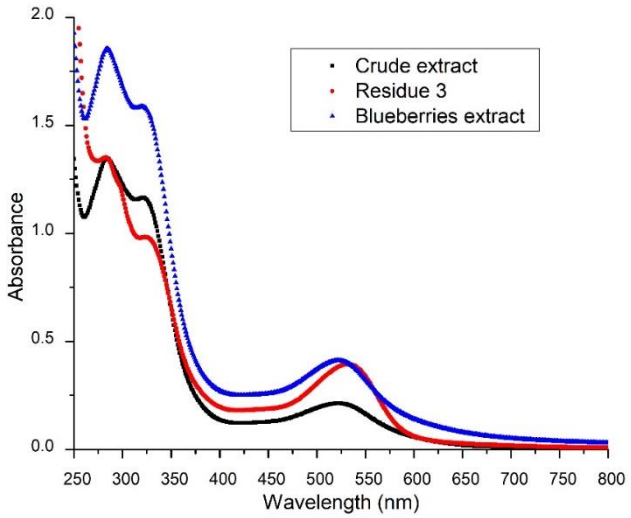

Figure 20: UV- analysis of Crude Extract, Residue 3 and Blueberries Extract.

The UV-Vis analysis was done with the purpose to establish the range of wavelength in which the anthocyanins, present in the extract, absorb UV-Vis spectrum. The three analyses were done in the range of 250 – 800 nm. This analysis was done for the Crude Extract, Residue 3 and Blueberries Extract and results in:

- *Crude Extract:* It was analyzed in the UV-Vis spectrometer showing a maximum peak at 284 nm with an absorbance of 1.3465 absorbance units (a.u.)

- *Residue 3:* It was analyzed by a UV-Vis spectrometer showing a maximum peak in the range 260 nm with an absorbance of 1.5851a.u.
- *Blueberries Extract:* The UV-Vis analysis shows a maximum peak at 284 nm with an absorbance of 1.8546 a.u.

UV-Vis spectroscopy analyses showed that the pigments of anthocyanins were not separated from other compounds present in the extract. It was assumed because the maximum peaks of the three analysis were found almost in the same region (**Figure 20**). The band at approximately 520 nm is attributed to the presence of anthocyanins that strongly absorb in the range 460-550 nm.[6,36,45]

The hypochromic effect, it is known that is a decrease in intensity occurred at 400-450 nm and then results in a hyperchromic effect with a medium intense absorption band in the range 450-550 nm.[37] Anthocyanin pigments are pH-dependent, and as a result, they can undergo reversible structural transformations, which are observed by absorbance spectra upon pH variations.[45] These transformations can be appreciated in the spectrum in **Figure 21**, obtained in the range 450-550 nm. The pH differential method allows a rapid evaluation of the total anthocyanins even in the presence of polymerized degraded pigments and other interfering compounds such as other phenolic compounds.

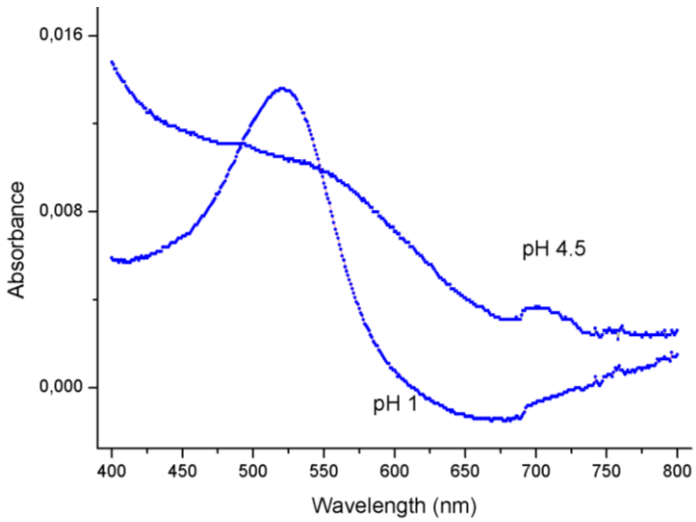

Figure 21: Spectral characteristic of Anthocyanins Extract at pH 1 and at pH 4.5

Literature reports that at pH 1.0 the colored oxonium form (ion which contains a trivalent oxygen atom[65]) predominates and at pH 4.5 the colorless hemiketal[6] form of the anthocyanins predominates (**Figure 22**).[45,66] Results indicated that anthocyanin pigments at pH 1.0 have an absorption peak at the range λ_{max} = 519-522 nm in the visible range. At

this same pH, anthocyanins mainly present as flavylium cations (oxonium ion) and the molecule is pigmented in red color.[66]

At pH 4.5, the absence of conjugated double bond between the second and the third aromatic ring in the carbinol form, where the hemiketal predominates, lead to the molecule loose its pigmentation and does not allow it to absorb visible light.[36,66]

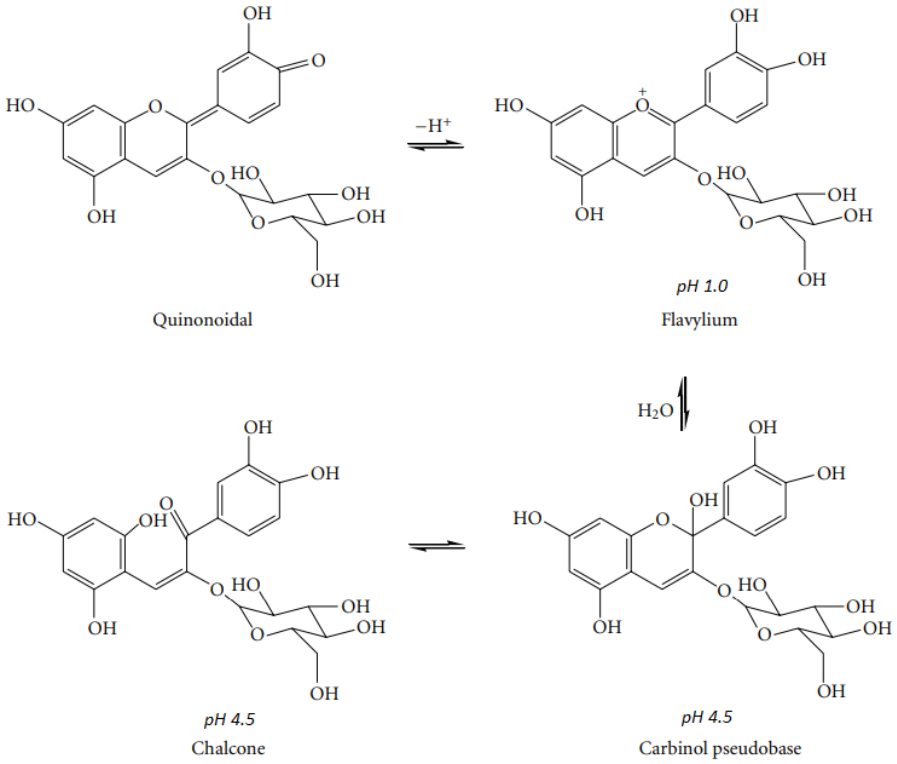

Figure 22: Various chemical form of cyanidin-3-glucoside. Buraidah, M. H. et al. (2001).[66]

However, as it was mentioned before, anthocyanins can be selectively detected at 520nm which correspond to the most common anthocyanin pigment found in nature, cyanidin-3-glucoside, whose structure is represented in **Figure 23**.[6,36,45,66] It should be mentioned that the glucoside of cyanidin-3-glucoside corresponds to galactose.[6]

Then, Using the Equation 4 and Equation 5, the result obtained in this UV-Vis analysis suggests that in the obtained Anthocyanin Extract, the content of cyanidin-3-glucoside in the extract was equal to $5093.16\,\frac{mg}{L}$. In the literature, the pigment content can be found in the range of 0.25 to $4.95\,\frac{\mathrm{mg}}{\mathrm{g}}$.[40] The total anthocyanins obtained in the laboratory expressed as $\frac{\mathrm{mg}}{\mathrm{g}}$ is equal to $4.95\,\frac{\mathrm{mg}}{\mathrm{g}}$. This comparison means that the value is in the upper limit and shows that the extraction was effective and high yield.[45]

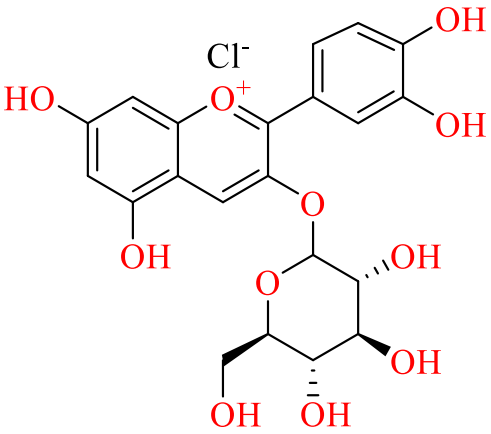

Figure 23: Cyanidin-3- glucoside structure.

4.1.4. Infrared Spectroscopy analysis

Infrared Spectroscopy is a technique used to identify functional groups[1], since each functional group absorbs radiation in a specific frequency of the infrared spectrum.[36] Since, using UV analysis, it was found that the anthocyanin extracted belongs to the cyanidin-3-glucoside molecule, a previous analysis of all possible vibration bonds implied in this molecule was performed in order to associate it to the experimentally obtained IR spectrum. **Table 6** summarizes the main IR peak frequencies of cyanidin-3-glucoside.

Table 6: IR assignments of cyanidin-3-glucoside.[36,37,67]

Wavelength (cm^{-1})	Assignments
3423 – 3370	Stretching vibration of O-H bond
2924 - 2855	Stretching vibration methylene group (-CH_2-)
1720 - 1708	C=O stretching band
1638 and 1444	C=C ring stretch absorption
1440 - 1220	C-O-H bending vibration
1300 -1000	Bending vibration of C-O bond
1260 - 1000	Stretching vibration of C-O bond
900 - 690	Ring substitution pattern out of plane bending

The experimental IR spectrum of Anthocyanin Extract showed in **Figure 24** is compared with the summarize analysis above.

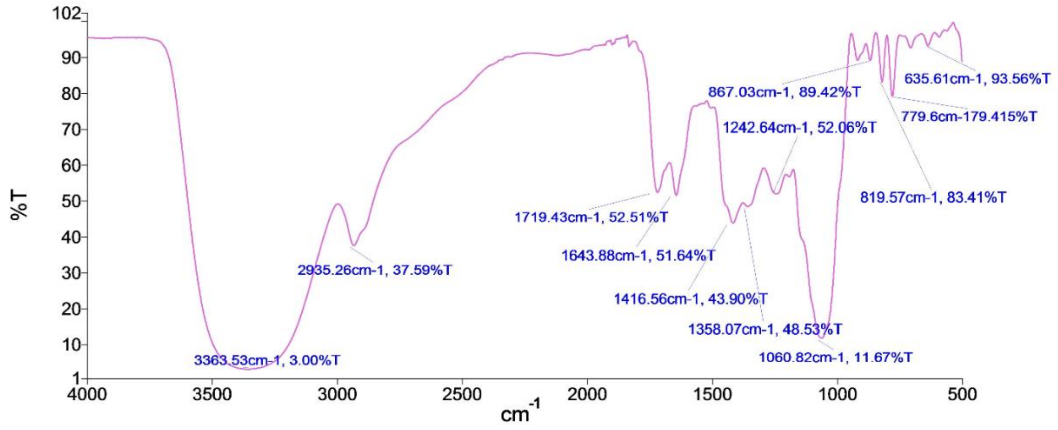

Figure 24: Infrared Spectrum of Anthocyanin extract.

IR spectrum of cyanidin-3-glucoside using a PerkinElmer Spectrum is illustrated in **Figure 24** showing the prominent vibration bands. Anthocyanin Extract showed a strong and broad absorption band belonging to the alcohol group attached to an aromatic ring and a glycoside structure at 3363 cm^{-1} (O-H stretch, H-bonded).[36,37,67] Stretching band at 2935 cm^{-1} belongs to the methylene groups (C-H sp^3) that can be found in the galactose structure of cyanidin-3-glucoside. At 1719 cm^{-1} there is a stretching band which can be attributed to C=O bond. C=C of aromatic ring and benzopyran (chromene)[36,37,67] present stretch absorptions occur in pairs at 1643 cm^{-1} and 1416 cm^{-1}. The absorption band at 1358 cm^{-1} may be attributed to the O–H in plane deformation in polyphenols whilst at 1242 cm^{-1} there is a stretching band that belongs to C-O of alcohols. At 1060 cm^{-1} is observed a band corresponding to bending vibration of C-O-C groups in the glycoside which corresponds to an ether; this band along with C=O band indicate the presence of carbohydrates.[36] Additionally, IR bands in the area from 900 to 690 cm^{-1} are attributed to bending ring vibrations out of the plane from the substitution of the aromatic rings.[36,37] Then, the IR analysis confirms the presence of cyanidin-3-glucoside in Anthocyanin Extract.

4.2. EVALUATION OF ANTIOXIDANT CAPACITY OF BLUEBERRIES EXTRACT

4.2.1. Total phenolic content of the extract

For this calculation, values of gallic acid used as standard were taken from University of Yachay Tech.[68,69,70] The department establishes three types of solution were used: a)

gallic acid stock solution, b) Folin-Ciocalteau Reagent and c) Sodium carbonate solution. To construct the calibration curve, gallic acid was used in different concentrations: 0 (blank solution), 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.2, 0.3 mg/mL. 300 μL of each calibration solution was mixed with 150 µL of 10% Folin-Ciocalteau reagent and then 120 µL of 7.5% Na_2CO_3. The solutions were incubated at room temperature for 30 minutes and measured the absorbance at 765 nm against the blank.[68] **Figure 25** shows the calibration curve of gallic acid at different concentrations from University of Yachay Tech, using the conditions of Department of Viticulture & Enology in the University of California, Davis.[68,69,70]

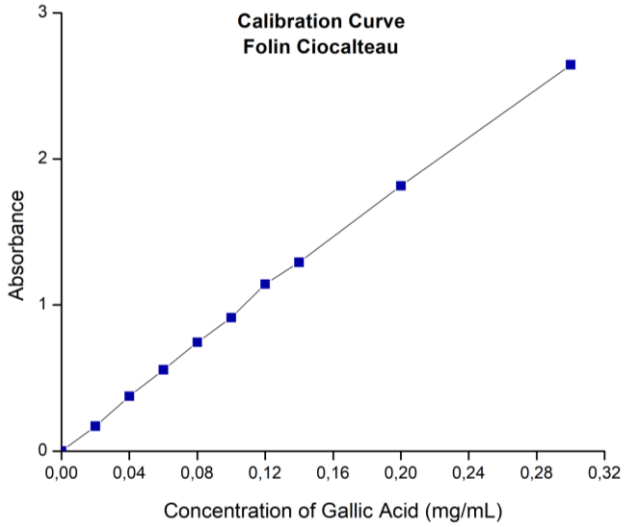

Figure 25: Calibration curve of Folin Ciocalteau –Gallic Acid at 765 nm. Yachay Tech University (2019).[68,70]

Quantification was done based on a calibration curve of gallic acid showed in **Figure 25** and its tendency line equation is $y = 8.8282x + 0.0328$ with R^2= 0.9987 and it was calculated with Microsoft Excel 2007 program. In this work, the analysis for the total phenolic content from Anthocyanin Extract yielded an absorbance at 760 nm = 0.9385. With an extrapolation of the equation of the line, using the value of absorbance obtained of the Anthocyanin Extract as "Y" value, the concentration of gallic acid related to the extract was 0.10 mg/mL gallic acid.

The result of approximate total phenolic content using Equation 2 for Anthocyanin Extract used in this analysis was 7.87 $\frac{\mu g}{mg}\frac{GAE}{EDW}$. Huang, W. et. al[71] establish that the total phenolic content of blueberries is 9.44 $\frac{\mu g}{mg}\frac{GAE}{EDW}$. The obtained concentration is lower

compared with what is reported in the literature that could be due to the concentration of the solvents used for the analysis.

4.2.2. Analysis of total flavonoid content of Anthocyanin Extract

Several studies reported that flavonoids contribute to antioxidant properties. This analysis was based on the aluminum chloride colorimetric assay. The values of absorbance obtained during the analysis at different wavelength were 11.93 at 420 nm and 7.212 at 700 nm. It means that at both wavelength, the extract was absorbing in aluminum chloride solution. However, studies have shown that flavonoids absorb in the range between 415-420 nm, so the absorbance of interest for this analysis was 11.93 and further, it is going to be used as the "Y" value.

Quantification of quercetin was taken from a study of Flavonoids and Phenolic Compounds from Litsea polyantha Juss. Bark.[72] At X axis the tested concentrations of quercetin (mg/L) was plotted while in Y axis the absorbance values at 415 nm was plotted.

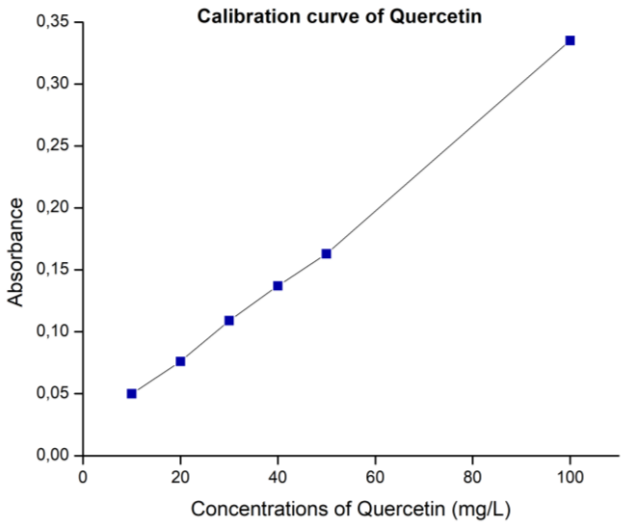

Figure 26: Calibration curve of quercetin for determination of total flavonoid content. Ghosh et al. (2014).[72]

Using the equation of line $y = 0.0031x + 0.0159$, as is shown in **Figure 26** and with a extrapolation of the equation, the concentration of quercetin related to the Anthocyanin Extract was 3843.25 mg/L. The approximate total flavonoid content for Anthocyanin Extract using Equation 3 was 7.68 $\frac{ug}{mg}\frac{QE}{EDW}$. This value is lower with the reported in the literature, 36.08 $\frac{\mu g}{mg}\frac{GAE}{EDW}$, the reasons for differences can be attributed to geographical and

climatic conditions that infer in concentrations of bioactive compounds in plants and their bioactivity for human health.[73]

4.2.3. Reducing power analysis of Anthocyanin Extract

Table 7: Absorbance results of Anthocyanin Extract and ascorbic acid.

% Concentration (μL/mL)	Absorbance at 700 nm	
	[Anthocyanin Extract]	[Ascorbic Acid]
10	0.283	0.688
15	0.409	0.936
20	0.437	1.234
25	0.706	1.468
30	0.788	1.523

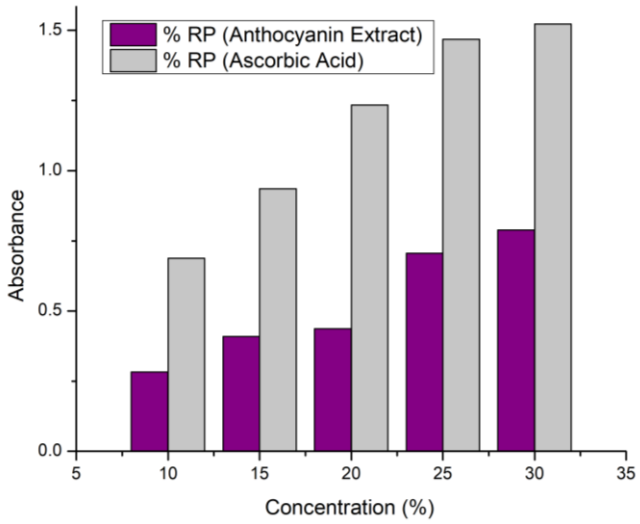

Figure 27: Reducing powder of Anthocyanin Extract and ascorbic acid at 700 nm. RP: Reducing Power.

The analysis was carried out using potassium ferricyanide $K_3Fe(CN)_6$ (1%) to know the reduction capacity of the Anthocyanin Extract. It was observed that all water-anthocyanin extracts were able to reduce the potassium ferricyanide. Considering the oxidation states of iron +2,+3, +4, +6,[74] the reaction must be a reduction from $K_3Fe(CN)_6 \rightarrow K_4Fe(CN)_6$. According to literature, the reduction reaction proposed is, Fe^{3+} to Fe^{2+}.[75,76]

Vitamin C or ascorbic acid was used as reference because it enhances iron absorption by reducing Fe^{3+} to Fe^{2+} from non-heme iron sources.[75,76] In accordance with Pehlivan, F.[75,76], ascorbic acid acts as a pro-oxidant in vitro agent[76] in the presence of redox ions

such as iron or copper inducing the oxidative stress contributing to the oxidation of DNA, lipid or proteins. However, Vitamin C can both act as a strong antioxidant agent and, at the same time, behave as a radical promoter at high concentrations in presence of transition metals.[75] Antioxidant compounds like ascorbic acid inhibit their reaction with biological sites due to its ability to donate electrons from its second and third carbon that can be identified in **Figure 28**, turning ascorbic acid in a potent reducing agent and scavenger of free radicals in biological systems.[9,75]

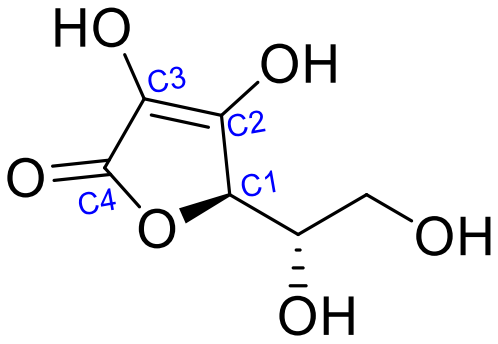

Figure 28: Molecular structure of ascorbic acid.

Anthocyanins have a high reducing power so they can be considered as powerful antioxidants,[77] although, in the reducing power analysis, the results suggest that water-anthocyanin extracts had less effective reducing power than vitamin C for potassium ferricyanide assay. This can be appreciated in **Table 7** and **Figure 27**. Also, the results obtained indicate that the reducing power of both, Anthocyanin Extract and vitamin C, increased with increasing concentrations as is represented in **Figure 27** and **Figure 29**.

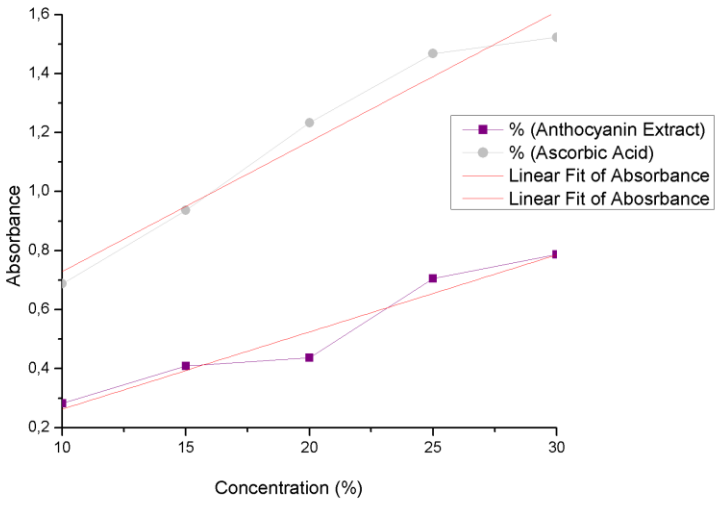

Figure 29: Reducing power of Anthocyanin Extract and ascorbic acid.

Table 8: Statistic analysis of reducing power results of Anthocyanin Extract and ascorbic acid.

Equation	y = a + bx			
	Anthocyanin Extract	Standard Error of Anthocyanin Extract	Ascorbic Acid	Standard Error of Ascorbic Acid
Adj. R-Square	0.92024	-	0.94792	-
Intercept	0.0018	0.08075	0.289	0.10874
Slope	0.02614	0.00381	0.04404	0.00513

Reducing power assay was a convenient and rapid screening method for determining the antioxidant potential. In **Figure 29** and **Table 8**, the reducing power of both compounds increased significantly showing a direct correlation between the concentration and absorbance of both ascorbic acid and Anthocyanin Extract. The obtained correlation coefficient R^2 was 0.92024 for Anthocyanin Extract and 0.94792 for ascorbic acid. From the results, it was possible to determine the IC_{50} index, which, for this case, represents the concentration of the extract at which 50% of the reducing activity is observed. The values for IC_{50} for Anthocyanin Extract were 19.05 $\frac{\mu g}{mL}$ and 4.79 $\frac{\mu g}{mL}$ for ascorbic acid.

Commonly, ferric chloride in solution is used as a color change indicator of the solution under study. In this case, **Figure 30** shows that the intensity of the color change depending on the concentration of the Anthocyanin Extract and ascorbic acid as a standard reference. In this project, $FeCl_3 \cdot 6H_2O$ (0.17%) was used to confirm the presence of organic derivatives and allowed to compare the ferric reducing ability of the antioxidants used in the present study.[78,79]

Figure 30: Solutions for Reducing Power Determination of Ascorbic Acid aqueous solution (Aa, Ab, Ac, Ad, Ae at different concentrations (10, 15, 20, 25 and 30%)) and Anthocyanin Extract aqueous solution (Ea, Eb, Ec, Ed, Ee at different concentrations (10, 15, 20, 25 and 30%)) prepared in the laboratory at Yachay Tech University.

4.2.4. Analysis of hydrogen peroxide assay for Blueberries Extract

In accordance with the methodology applied for this assay, the ability of Anthocyanin Extract to eliminate hydrogen peroxide is given by its antioxidant property. The obtained results for this analysis using Equation 6 at 560 nm were: $A^o = 0.2696$ and $A = 0.02064$. These results give an H_2O_2 scavenging activity mostly removing free radicals equal to 92.34%. This occurs through the donation of hydrogen to free radicals to convert them into nonreactive species.[48] Then, for this assay, Anthocyanin Extract was a powerful antioxidant scavenger of hydroxyl radical.

4.2.5. Analysis for Phosphomolybdenum assay

Table 9: Absorbance values for Anthocyanin Extract and ascorbic acid during phosphomolybdenum assay.

Phosphomolybdenum Assay	
Sample	**Absorbance at 695nm**
Anthocyanin extract	0.8456
Ascorbic Acid	0.4733

Table 9 shows that in the presence of Anthocyanin Extract, molybdenum is reduced more effectively at 695 nm. In literature, it is expected that absorbance values will increase as sample concentrations increase.[50,51] Meanwhile, in the wavelength region under study, the variation between the absorbance of blueberry extract and ascorbic acid is observed in the spectrum shown in **Figure 31**, along with the comparison with molybdenum reagent used as blank.

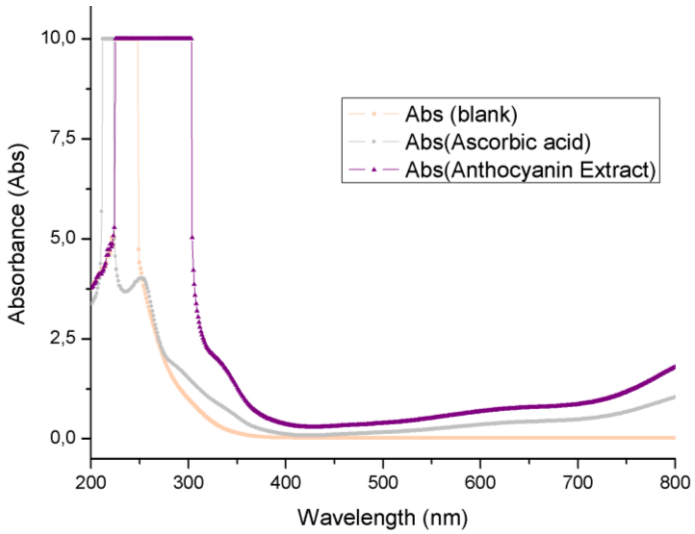

Figure 31: Ultraviolet spectrum of molybdate reagent with Anthocyanin Extract (purple color), molybdate reagent with ascorbic acid (gray color) and blank as molybdate reagent (salmon color).

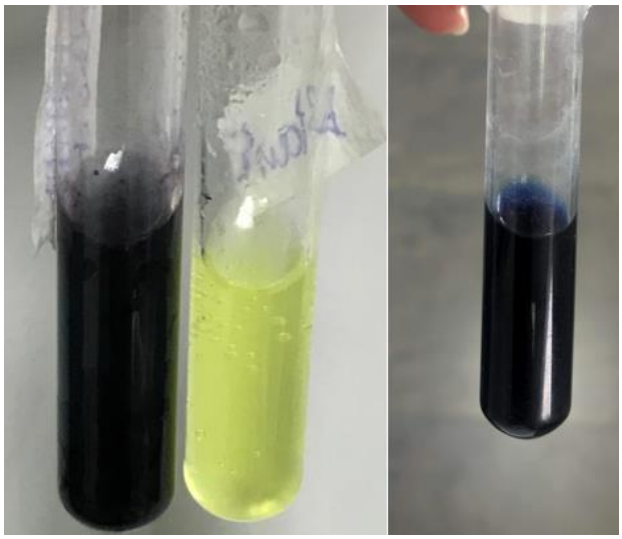

Figure 32: Test tubes for phosphomolybdenum assay. Test tube of the left: molybdate reagent with Blueberries Extract, test tube of the middle: molybdate reagent and test tube of the right: molybdate reagent with ascorbic acid. Solutions were prepared in the laboratory at Yachay Tech University.

The reducing reaction can be seen in **Figure 32** noting the difference in color presented by the samples tested. The molybdenum reagent, initially green-yellow, changed to an intense blue color for the case of the reaction with ascorbic acid (rightmost tube), while for the sample corresponding to the Anthocyanin Extract, the final color became darker/more intense (leftmost tube). These results suggest that Blueberries Extract presents a higher reducing activity than ascorbic acid.

4.3. CHARACTERIZATION OF EMULSION

4.3.1. Results of the type of primary emulsions

After eight emulsion samples were prepared, the type of primary emulsion of each sample is detailed in **Table 10**. Once the dilution tests were carried out, W/O emulsions (V and VI) were dispersed in an oily medium, while they were immiscible in the aqueous medium. In contrast, O/W emulsions (I-III and VII-VIII) formed dispersions in an aqueous medium, being immiscible in the oily medium.

Regarding to O/W emulsions, the presence of considerable quantities of CTAB produces more stable O/W type emulsions.[27] The intention to use soy lecithin as a surfactant was based on its low HLB value, which would promote the formation of W/O emulsions according to Griffin scale.[27] Also, due to the lack of availability in the laboratory of lipophilic surfactants, the amphoteric character of lecithin was used under certain conditions as a lipophilic surfactant.

Table 10: Type of primary emulsion of the emulsion samples prepared in the laboratory at Yachay Tech.

N°. Emulsion	Type of Primary Emulsion
I	O/W
II	O/W
III	O/W
IV	O/W
V	W/O
VI	W/O
VII	O/W
VIII	O/W

Both types of primary emulsions were stable. Concerning volume, the most stable emulsions were O/W emulsions. The volume of O/W emulsions was reduced by 50% while W/O emulsions were reduced by 70% of their initial volume after two days. W/O emulsions presented bubbles after four days of preparing it while O/W emulsions did not present this characteristic. In addition, the texture of W/O emulsions was greasy as shown

in **Figure 33A** while the other O/W emulsions had a creamier texture as shown in **Figure 33B**.

The lower stability of the W/O emulsion compared to the O/W can be attributed to the amphoteric character of lecithin, which could eventually migrate to the aqueous phase, destabilizing the initially formed emulsion.[80] In addition, it would have been more convenient to use a hydrophilic surfactant with a lower HLB value, instead of the CTAB used, which has a moderate HLB value. As mentioned above, the reagents used were those available in the laboratory, or that were relatively easy to acquire.

On the other hand, mixing and stirring of both phases to form emulsions was carried out with a device not suitable (mini hand-mixer) for this purpose, since the required high rpm was not achieved. However, despite not being able to prepare highly stable emulsion, it was possible to understand and evaluate the system.

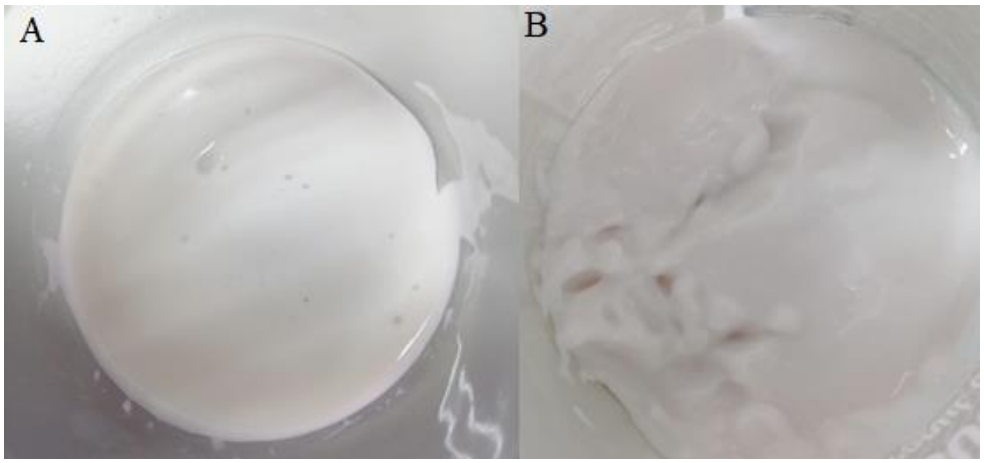

Figure 33: (A) W/O emulsion and (B) O/W emulsion prepared in the laboratory at Yachay Tech University.

4.3.2. Analysis of the conductivity on emulsions

W/O emulsions had a very low conductivity because the continuous phase, the external one, consisted of the oil phase. The conductivity increases for the case of O/W emulsions. This difference in electrical conductivities contributes to the O/W emulsion stability mechanism being different from that for W/O.[54] O/W type of emulsions are stabilized by steric and electrostatic repulsions, while for W/O emulsions, the steric force is the dominant force due to the low electrical conductivity.[54] After analyzing all samples obtained in the tests, those emulsions denoted as V and VI (highlighted in pink color in

Table 11) were selected as the most appropriate, due to their characteristics that reflected their W/O nature.

Table 11: Primary type sample emulsions with their respectively conductivity.

N°. Emulsion	Type of Primary Emulsion	Conductivity
I	O/W	388-389 μS
II	O/W	1493 – 1515 μS
III	O/W	322-331 μS
IV	O/W	3.05 ms
V	W/O	1.5 μS
VI	W/O	< 1 μS
VII	O/W	287μS
VIII	O/W	350 μS

4.3.3. Analysis of W/O/W emulsion

In the preparation of the double emulsion, adding W_2 to the primary emulsion (W_1/O) previously prepared, the emulsion was compacted in such a way that a homogeneous mixture was not achieved. The simultaneous application of heat and stirring caused both phases, W_1/O_1 and W_2, to mix successfully, however when the heat disappears, the mixture disappears, leaving both phases separated. The same goes for emulsion VI. In both composed emulsion W/O/W, the phase W_2 was composed of water and a small amount of lignin added. The double emulsion was prepared from primary emulsions V and VI and called sample A and sample B double emulsion. The conductivities for $W_1/O_1/W_2$ for samples A and B were 198 μS and 186.9 μS, respectively.

HLB of a mixture can be calculated through an Equation 7 combining the HLB of each component, an HLB of 4.5 and 10 for lecithin and CTAB respectively.

$$\textit{Equation 7: } HLB_{mixture} = (HLB_{lecithin} \times concentration\ of\ lecithin\ in\ the\ emulsion) + (HLB_{CTAB} \times concentration\ of\ CTAB\ in\ the\ emulsion)$$ [81]

This formula gives an HLB for mixture A of 0.31 and 0.15 for sample B concluding that both W/O/W emulsion are of lipophillic character.

Sample B W/O/W double emulsion was more stable than Sample A W/O/W double emulsion because was more compacted and the other showed lumps between its phases. This difference between A and B can be observed in **Figure 34**. The conditions for the preparation of the double emulsion were not optimal, as is recommended in the industry of cosmetics. For example, in this project, the stirring was developed with a mini hand-

mixer instead of an ultra-turrax recommended in the conventional formulation. Also, the type of surfactants available in the Laboratory of Chemistry was limited. Although the HLB factor resulting from the finally used surfactant was appropriate, it was not enough to generate more stable double W/O/W emulsions.

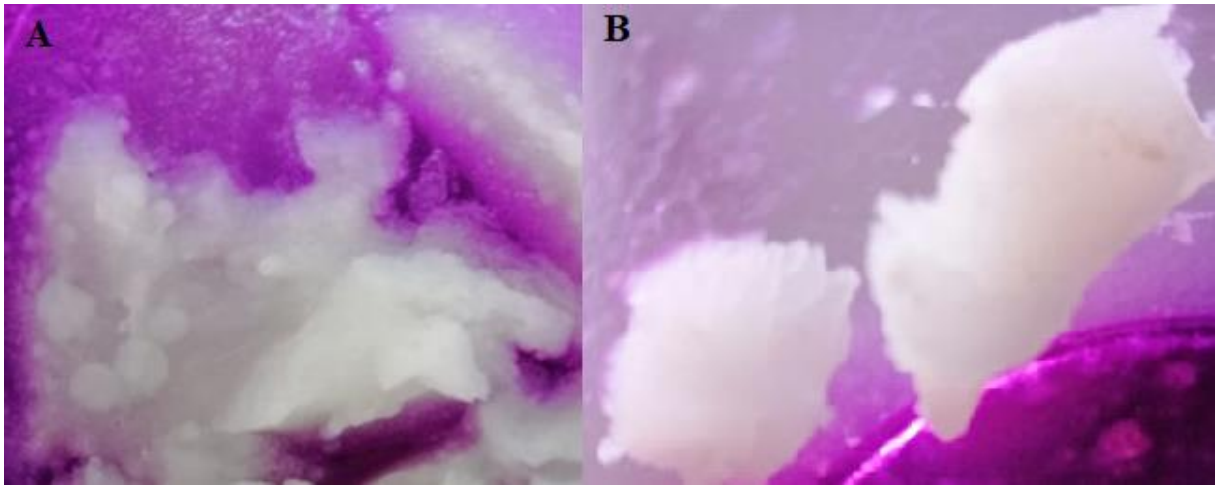

Figure 34: (A) Sample A W/O/W double emulsion and (B) Sample B W/O/W double emulsion prepared in the laboratory at Yachay Tech University.

4.3.4. Microscopy analysis of Anthocyanin Extract and emulsions

Optical visualization of extract at 10X and 20X showed anthocyanin pigments contained in a solution of formic acid:methanol:water with a ratio 3:14.55:82:45. The observed bubbles are the product of the same solution used to encapsulate the pigments. Visually, the shape of the pigments is elongated as can be seen in **Figure 35**, but it cannot be confirmed since this would require high-resolution equipment such as Scanning Electron Microscopy to produce high-resolution images of the surface of a sample.[82]

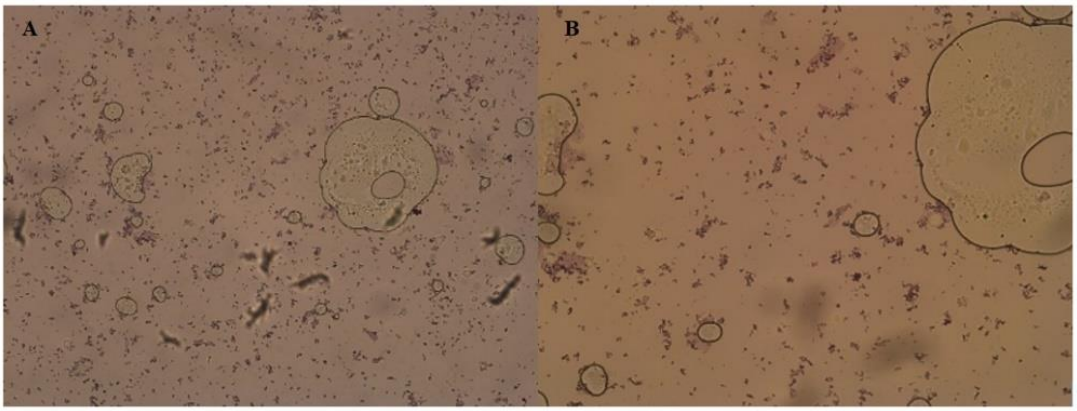

Figure 35: Optical visualization of the Anthocyanin Extract with microscopic lenses 10X (A) and 20X (B).

Optical visualization in **Figure 36** and **Figure 38** shows the droplets of primary emulsion W/O. These emulsions had oil as the continuous phase and as the dispersed phase, water with the extract. The colloidal particles formed to possess a thick layer due to the surfactants in which its hydrophilic head was inside the particle in contact with the aqueous phase, and the lipophilic (hydrophobic) tail is oriented towards the outside of the particle in contact with oil phase, as can be seen in **Figure 37** and **Figure 39**. So, geometric shapes of micellar aggregates could be elongated spherical. The exact size of the drop could not be taken because the equipment used did not have that a scale to measure, however, it was visually estimated that the size of the drop is between microns and millimeters.

The size of the micelles depends on the change in free energy, which is given by the self-association of amphiphilic (or amphipathic) molecules in a solution. Thus, the amphipathic molecules have a hydrophobic tail and a hydrophilic head and to form the micelles, there must be a force of repulsion between the polar head groups which limits the self-association of the aggregates and hydrophobic force which helps to the expulsion of the non-polar side of the amphiphilic molecule from the aqueous medium with the formation of a droplet or fluid organic droplet that constitutes the micelle core.[83]

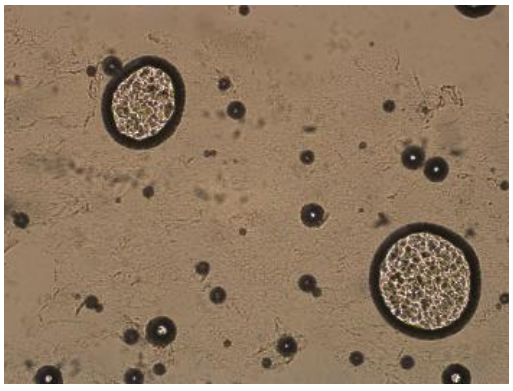

Figure 36: W/O emulsion of sample V with 10X microscope lenses.

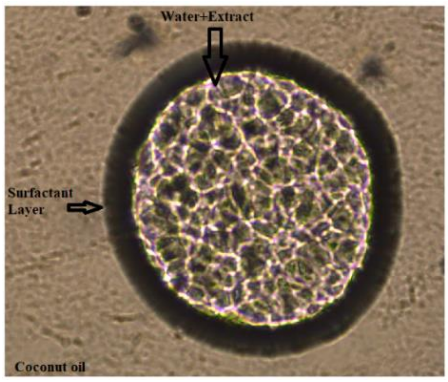

Figure 37: Micelle structure of W/O emulsion of sample V conditions.

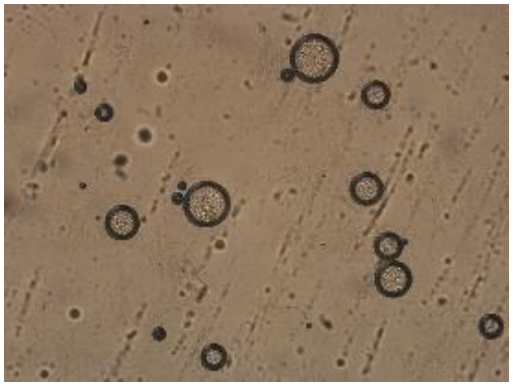

Figure 38: W/O emulsion of sample VI with 10X microscope lenses.

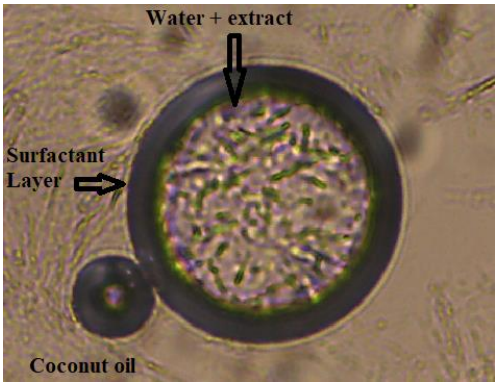

Figure 39: Micelle structure of W/O emulsion of sample V conditions.

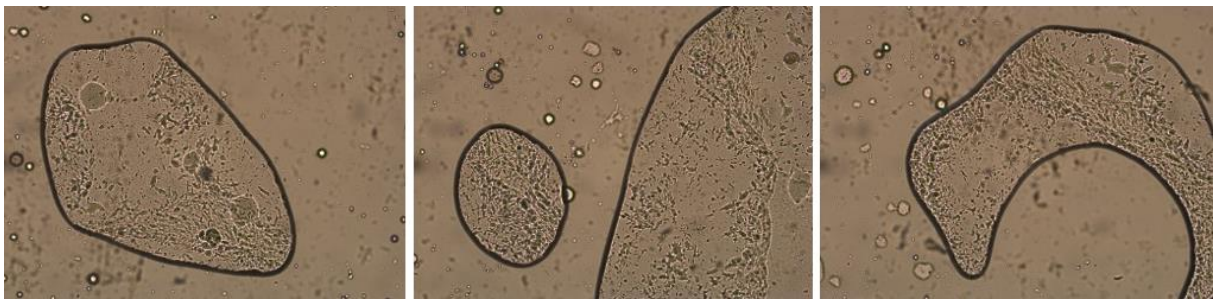

Figure 40: Double emulsion W/O/W from sample V primary emulsion visualized with 10X microscope lenses

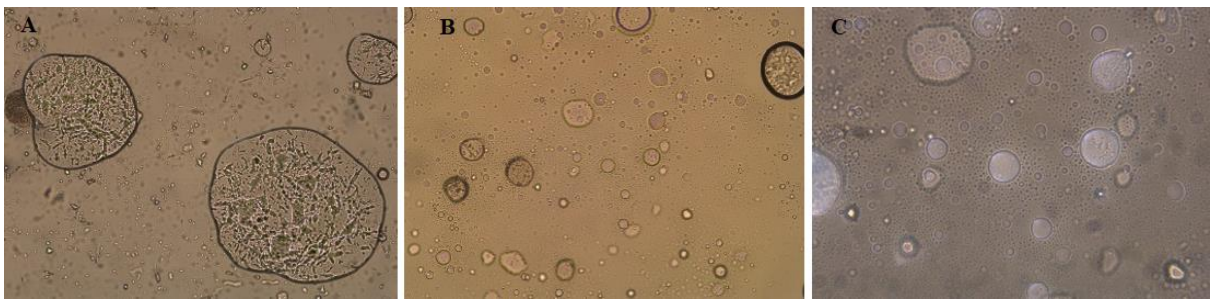

Figure 41: Double emulsion W/O/W from sample VI primary emulsion visualized with 10X (A), 20X (B) and 40X (C) microscope lenses.

In **Figure 42** and **Figure 43**, it is possible to see how the anthocyanin pigment is in the water phase inside the W/O/W emulsion (figures taken from **Figure 40** and **Figure 41**). Also, in both figures, it can be established that the double emulsions were formed.

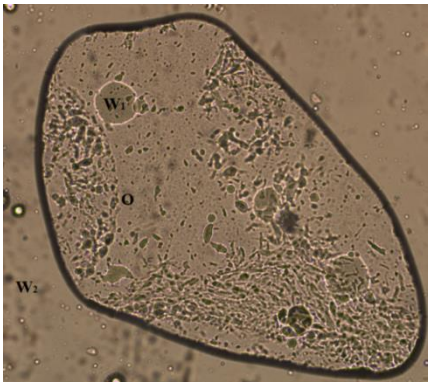

Figure 42: W/O/W emulsion (from sample V type primary emulsion) at 10X.

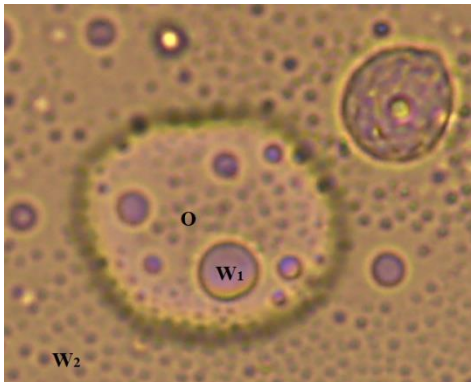

Figure 43: W/O/W emulsion (from sample VI type primary emulsion) at 20X.

The shape of the micelles is influenced by the surface area of an amphiphilic molecule: when this area decreases, there is an increase in the number of micelle aggregation.[83] The surface of the nucleus of the micelles present and irregular surface as they are in fluctuation because the hydrophobic part is liquid. Therefore, the particles undergo a Brownian motion causing crashes between particles inside the emulsion. These particle movements are incessant and random, and the particles continually change direction, generating a spatial distribution that is governed by the interactions present in both phases of the emulsion.[22]

The surfactant plays a major role in systems that have a continuous liquid phase in which the surfactant can migrate by convection or diffusion. At critical micelle concentration (CMC), the minimal concentration at which surfactant induces the formation of micelles spontaneously in solution and above that, the surfactant acts as pro-oxidants.[28] It has been reported that the CMC of lecithin used as a supplement is 1288 ppm[28] in oil. Therefore, the concentrations of lecithin in the preparation of emulsion were always lower than that value, using 0.56 ppm in the oil phase. Concerning CTAB, according to literature, CMC in water is 328 ppm.[84] According to this project, the concentration of CTAB in the water phase of prepared W/O/W emulsion was around 125 ppm, it means, it was below the reported CMC value.

4.4. UVA-UVB absorption capacity of Anthocyanin Extract and emulsions prepared

Ultraviolet radiation A (UVA (320-400nm[11,85])) has a longer wavelength than UVB and is associated with skin aging, while ultraviolet B (UVB (290-320nm[11,85])) is associated

with skin burning.[86] The UVA-UVB digital meter used gave a single measurement considering both wavelength ranges. It is known that UV rays tend to be partially absorbed blocked by glass material.[85] For this reason, in this project, initially clean Petri dishes were exposed at UV radiation from sunlight. The UVA-UVB radiation value recorded by the device was 1.18 $\frac{mW}{cm^2}$. Later, the Petri dishes were exposed to radiation emitted by a UV lamp Vilber Lourmat, N° 17100080 (radiation box) with a wavelength of 365 nm (UVA). In that case, the recorded measurement was 1.50 $\frac{mW}{cm^2}$.

Table 12: UVA-UVB values of Anthocyanin Extract taken on a day with little solar radiation and on a radiation box at 365 nm.

Concentrations of Anthocyanin Extract	UVA-UVB values ($\frac{mW}{cm^2}$) with solar radiation at 13:51 pm	UVA-UVB values ($\frac{mW}{cm^2}$) on a radiation box
Anthocyanin Extract	0.22	0.43

Because of anthocyanins and polyphenolic compounds have the ability to act as broad-spectrum sunscreens, covering UVA and UVB ranges.[87] The UV absorption capacity by measures of how much UV radiation these phenolic compound absorb using the mentioned device. The values are shown in **Table 12** and correspond to 0.22 $\frac{mW}{cm^2}$ for that simple exposed to the sun, and 0.43 $\frac{mW}{cm^2}$ for that exposed to radiation box. These values represent a difference in absorption of 0.96 $\frac{mW}{cm^2}$ and 1.07 $\frac{mW}{cm^2}$, corresponding to 81.35% and 71.33% of absorption capacity, respectively. So, in addition to the antioxidant nature of the extract, it exhibits properties in ultraviolet absorption.

Table 13: UVA-UVB values of Anthocyanin Extract taken on a radiation box at 365 nm.

N° sample of W/O/W double emulsion	UVA-UVB values ($\frac{mW}{cm^2}$)
A	1.08
B	0.93

In W/O, O/W, W/O/W, O/W/O systems, it is the oil that provides UV absorption.[85] In addition, the oil contained in the lipophillic phase, the Anthocyanin Extract absorbs also partially the UV radiation. The results suggest that there is a synergy between the characteristics of the emulsion and the extract, giving 0.42 $\frac{mW}{cm^2}$ for double emulsion A and 0.57 $\frac{mW}{cm^2}$ for double emulsion B obtained from the difference between the value of

radiation box and values of **Table 13** giving an absorption capacity of 28% for double emulsion A and 38% for double emulsion B. Lastly, antioxidants have anti-radical properties, protecting skin tissue from aging and photoinduced skin cancers due to long periods of sunlight exposure.

4.5. Moisturizing analysis of emulsions

The use of oils prevents the loss of water in the skin forming an epicutaneous lipid film.[20] In prepared emulsions, coconut oil was in the oil phase. This oil has moisturizing, antibacterial and antioxidant properties. These properties help to skin body to absorb the vitamins (E and K) and minerals necessary to provide softness and firmness in the skin.[88]

Coconut oil is recommended for both the skin of the body and the skin of the face. However, special care of the face should be taken into account as it is a particularly delicate part. Oily or mixed (mixed skin has oily and dry zones) facial skin has a main characteristic based on the excessive production of sebum to counteract the lack of hydration. Excessive sebum production is due to open pores, which can produce pustules filled with pus in the presence of environmental pollutants and hormonal changes.[1,89] The treatment for these types of skin with W/O and W/O/W emulsions with greasy texture is not a good combination because the oil irritates the existing pimples and promotes the appearance of many more pimples. The greasy texture of these emulsions is attributed to the conditions in which they were prepared, that is, the oil concentrations were higher than the water.

Emulsions were tested on the heel skin of the foot of a person with 0% moisture indicating that the skin is hydropenic, this value was obtained with a *Digital Moisture Monitor for Skin*. The person who underwent this assay was a 22-year-old woman with mixed skin. The double W/O/W emulsions gave an appearance of hydrated skin for 5 hours while the simple emulsion W/O gives an appearance of hydrated skin for 4.5 hours. However, the appearance of the skin does not justify how much moisturized the skin was since it was only a qualitative test as can be seen in **Figure 44**. Then double W/O/W emulsion containing 5% blueberry extract could increase the water content in the stratum corneum for approximately 5 hours.

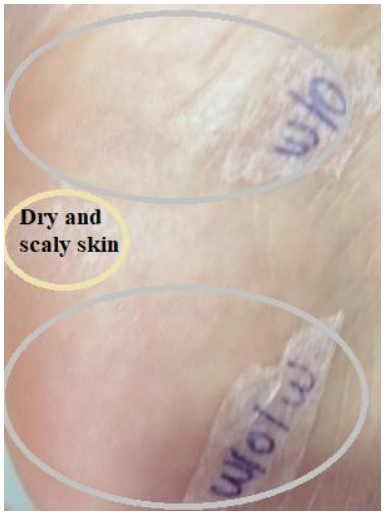

Figure 44: Hydration of the skin with emulsions W/O/W and W/O during 5 hours.

On the other hand, both double emulsions W/O/W were evaluated in the arm of the same person comparing the obtained values with a commercial emulsion. The skin of the arm has a moisture range between 29-35.3% demonstrating that the skin of hydropenic type according to the values of the moisture monitor based on **Annex 3** in **Table 19**. During 3 hours, the moisture of skin with emulsions decreases significantly as shown in **Figure 45**. This indicates that not even commercial creams provide much hydration to the skin for more than 3 hours.

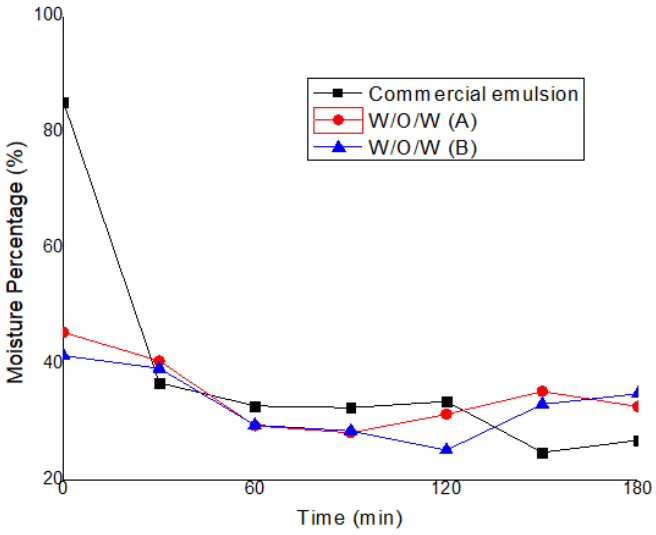

Figure 45: Moisture percentage of commercial emulsion and both double emulsions W/O/W prepared at Yachay Tech Laboratory.

Furthermore, in **Figure 45** can be seen that the double emulsion maintains a moisturizing level with respect to the commercial emulsion, this makes, in a certain manner, the double

emulsions are more effective in providing moisture to the skin. The oils are essential within an emulsion since its function is to provide moisturizing to the skin. Moisturizers, prepared as emulsions can act by an occlusive mechanism,[20] which alters the evaporation of moisture at the surface of the skin by forming an epicutaneous lipid film.

4.5.1. Antioxidant properties of Anthocyanin Extract

The antioxidant property of the Anthocyanin Extract was analyzed to treat the spots on the skin. However, this was only done with the extract since the most delicate spots were on the face. The person has a facial skin of hydropenic type with 18.3% of moisture based on **Annex 3** in **Table 19**. The person used the concentrated extract for two weeks. The initial spots were very dark because of the important melanin production in this person was too high causing dark spots at the height of the right cheekbone of the face. In **Figure 46**, the evolution of the spots is shown (A = spots before using the extract of blueberries, B = spots with the anointed extract, C= final version of treated skin after two weeks of use). The extract was gradually applied, each night to analyze if the skin suffered an allergic reaction. First, the extract was placed on the skin for two hours for two days in a row without showing an allergic reaction. Second, the extract was placed on the skin for four hours for three days showing healing of small wounds. The rest of the days until the end of the two weeks, the small scars changed the scab and the skin became smooth and finally, the spots were reduced by 90%.

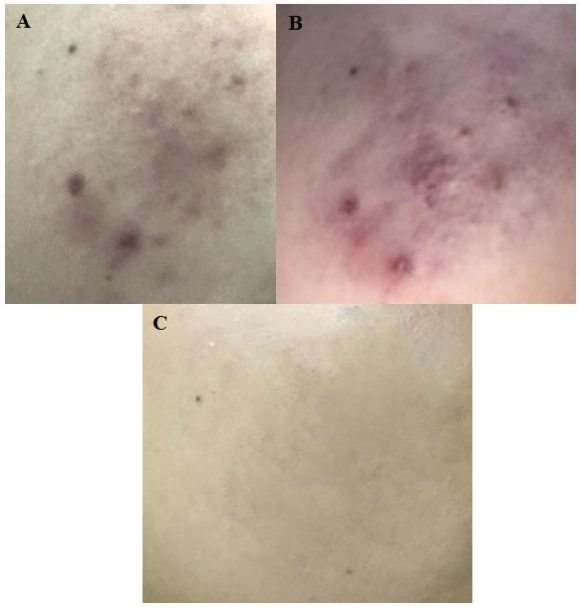

Figure 46: Evolution of acne spots in the patient. A= spots before using the extract of blueberries, B= spots with the anointed extract, C= final version of treated skin after two weeks of use.

Face spots (**Figure 46A**) are the product of acne exposed to various environmental factors. Pimples or also called imperfections in the skin usually leave marks of post-inflammatory hyperpigmentation because they occur once the inflammation itself decreases. When the skin tissue is damaged, as is the case with acne pimples, an excessive amount of melanin can accumulate in one area. This excess melanin remains in the form of spots once the imperfections have healed leaves its mark on the skin.[1,16]

The reduction of skin blemishes is probably due to the powerful antioxidant contained in the extract. This antioxidant softened the skin in the area that was treated and it was confirmed that Blueberries Extract is a powerful antioxidant for skin due to its content of vitamin C.[75] Generally, antioxidants help to the stimulation of collagen where it is the protein that prevents the appearance of wrinkles and improves skin elasticity.[90]

5. CHAPTER 5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

- It was possible to obtain Anthocyanin Extract from blueberry fruits, which was evidenced by the appearance, spectroscopic analysis, and chemical test, however, its identification of the components was not possible.

- The antioxidant capacity of the extract obtained through the development of different tests established in the literature was evidenced.

- It was possible to prepare W/O and W/O/W emulsions, in which the blueberry extract was encapsulated. Employing an appropriate identification test, it was possible to establish the type of emulsions obtained.

- Unfortunately, appropriate stability of the prepared W/O/W emulsion was not achieved, because suitable surfactants were not available in the laboratory and their acquisition was not possible.

- The capacity of absorption of UVA and UVB radiation of the extract of blueberries and prepared emulsions were verified, showing the synergy between the effects of their components. The double emulsion B with the highest content of Blueberries Extract presented a 38% absorption of UV radiation, 10% above that exhibited by the double emulsion A.

- The antioxidant property of blueberry extract showed that it has an effect to treat facial skin blemishes. This implies that emulsions prepared from this extract can treat spots on the body. It means, that both the extract and the emulsions have the power to reduce and treat the consequences on the skin caused by oxidative stress.

- The samples showed effectiveness in providing moisture to the skin.

5.2. Recommendations

- In the TLC analysis and purification Chromatography Column according to literature, the best way for separate and fractionate anthocyanins is using Reverse Phase Silica. It retains non-polar compounds due to strong hydrophobic interactions.[18,91] It means that in an aqueous phase, anthocyanins pigments are located in the solid phase, while acids and

sugars which are polar compounds can be washed away with acidified water and ethyl acetate for polyphenols compounds.[18]

- An ultra turrax to prepare the emulsions will give them greater homogenization because an electric hand mixer may not have the revolutions necessary for mixing.

- The use of appropriate surfactants such as the most commonly used in cosmetics: carboxylic acids, sulfonic acid derivatives, sulfuric acid derivatives, phosphoric acid derivatives, alkyl amines, alkyl imidazolines, alkyl betaines, heterocyclic ammonium salts, alcohol, esters, ethers, amine oxides, polymers and among others.[92]

- It is recommended to make an analysis using a Scanning Electron Microscope to visualize the extract and the emulsions, this will help confirm the shape of the anthocyanins in the extract and the size of the drops in the emulsions.

- It is recommended to do an appropriate analysis for the commercial application of emulsions in the cosmetic area such as rheology, sensory and small-angle X-ray scattering analysis.[93] Rheology measurement methods to study the flow and deformation of fluids exposing the samples to stress conditions, so this analysis can be done using: capillary viscometer, orifice viscometer, rotating spindle viscometer, rotating cylinder viscometer, creep measurement, skin viscoelasticity meter, among others.[1] Additionally, sensory measurements include storage, packaging, and maintenance of the emulsions. And finally, small-angle X-ray scattering analysis is used in order to evaluate the presence of liquid crystalline structures which commonly are used to increase the stability of the formulations.[1,93]

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ANNEXES

Annex 1.

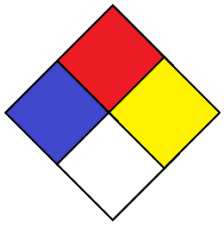


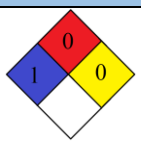
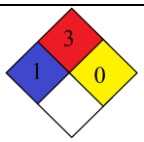
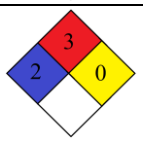
Illustration 1: Pictogram – Safety Rhombus

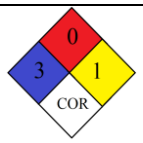
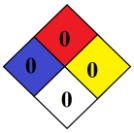
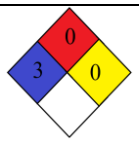
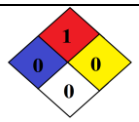
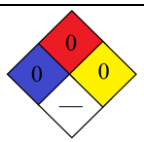
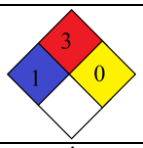
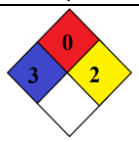
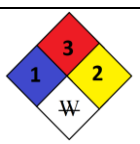
Table 14: Description of pictogram

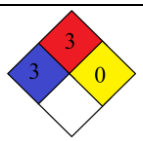
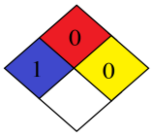
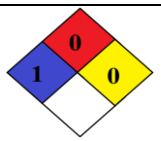
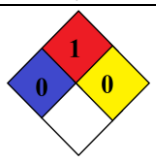
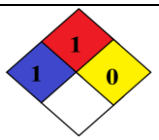
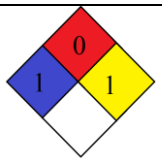
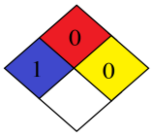
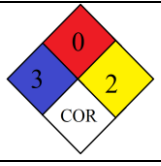
Risk Level	**Inflammability**	**Reactivity**	**Specific Risk**
4 – Mortal 3 – Very Dangerous 2 – Dangerous 1 – Little Dangerous 0 – Risk Free	4 – Below 25°C 3 – Below 37°C 2 – Below 93°C 1 – About 93°C 0 – Does not ignite	4 – May suddenly explode 3 – May explode in case of shock or heating 2 – Unstable in case of violent chemical change 1 – Unstable in case of heating 0 – Stable	OX – Oxidizer COR – Corrosive –Radioactive W – Do not use water – Biological Risk

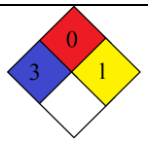
The safety rhombus is a symbol used internationally to indicate the level of risk that a substance can represent for human safety and health. According to this, in **Table 15** are described the reagents used in the laboratory with their respective safety rhombus.

Table 15: Physical and Chemical properties of reagents used in the extraction and characterization of blueberries and preparation and characterization of emulsions.

Reagent (Chemical Name)	Chemical Formula	Molecular Mass	Density	Boiling Point	Melting Point	Toxicity
Acetic Acid 0.2M	CH3COOH	60.05 g/mol	1.05 g/cm3	118°C	17°C	
Acetone	C3H6O	58.08 g/mol	0.79 g/cm3	56°C	-95°C	
Acetonitrile	C2H3N	41.05 g/mol	0.78 g/cm3	82°C	-45°C	

Acid chloride	HCl	36.46 g/mol	1.12 g/cm3	48°C	-26°C	
Aluminum chloride	AlCl3	133.24 g/mol	0.00248 g/cm3	180°C	192.4°C	
Ammonium molybdate	(NH4)2MoO4	196 g/mol	Not available	Not available	190°C	
Ascorbic acid	C6H8O6	176.13 g/mol	1.65 g/cm3	Not available	190°C	
Cetyl Trimethyl Ammonium Bromide	C19H42BrN	364.45 g/mol	0.5 g/cm3	Not available	237-243°C	Not available
Chloroform	CHCl3	119.38 g/mol	1.49 g/cm3	61°C	-64°C	
Coconut Oil			0.541- 0.619 g/cm3	260°C	23°C	
Distilled water	H2O	18 g/mol	0.99 g/cm3	100°C	0°C	
Ethyl acetate	C4H8O2	88.10 g/mol	0.9 g/cm3	77.1°C	-84°C	
Ferric chloride hexahydrate	FeCl3*6H2O	270.30 g/mol	1.66 g/cm3	280-285°C	37°C	
Folin-Ciocalteau reagent	Not available	Not available	1.20 g/cm3	100°C	-17.5 to 21.1°C	Not available
Formic Acid	CH2O2	46.03 g/mol	1.22 g/cm3	101°C	8°C	

Glycerin	C3H8O3	92.09 g/mol	1.26 g/cm3	290°C	18°C	
Hydrogen peroxide	H2O2	34 g/mol	1.45 g/cm3	150°C	-1°C	
Lignin	Not available	Not available	1.39 g/cm3	Not available	Not available	Not available
Methanol	CH3OH	32.04 g/mol	0.79 g/cm3	65°C	-97°C	
Phosphate buffer (pH 7.2)	Not available	Not available	1 g/cm3	Not available	0°C	
Potassium chloride	KCl	74.55 g/mol	1.98 g/cm3	1420°C	776°C	
Potassium ferricyanide	K3Fe(CN)6	329.24 g/mol	1.89 g/cm3	Not available	Not available	
Propylene glycol	C3H8O2	76.09 g/mol	1.13 g/cm3	188.2°C	-59°C	
Sodium acetate	CH3COONa	82.03 g/mol	1.45 g/cm3	881°C	324°C	
Sodium carbonate	Na2CO3	105.98 g/mol	2.54 g/cm3	1600°C	851°C	
Sodium phosphate	Na3PO4	163.94 g/mol	Not available	Near to 100°C	Near to °OC	
Sulfuric acid	H2SO4	98.08 g/mol	1.83 g/cm3	337°C	10°C	

Trichloroacetic acid 99%	C2HCl3O2	163.38 g/mol	1.63 g/cm3	197°C	58°C	

Annex 2.

Table 16: List of chemical reagents and raw materials used in the development of this project.

Chemical Reagent	Description
Acetic Acid 30%	Fisher Scientific at United States of America
Ascorbic Acid	Spectrum, Chemical MFG Corp. at Gardena, California
Formic Acid	Loba Chemie Laboratory – India
Hydrochloric Acid	Fisher Scientific at United States of America
Sulfuric Acid 95-97%	EMSURE – Merck kGaA at Germany
Trichloroacetic acid	Loba Chemie Laboratory – India
Acetone	Fisher Scientific at United States of America
Acetonitrile	Fisher Scientific at United States of America
Chloroform	Loba Chemie Laboratory – India
Ethyl acetate	Fisher Scientific at United States of America
Methanol	EMSURE – Merck kGaA at Germany
Aluminum chloride 98%	Sigma – Aldrich, Merck kGaA at Germany
Ammonium molybdate	Loba Chemie Laboratory – India
Ferric chloride hexahydrate	Loba Chemie Laboratory – India
Potassium chloride	Fisher Scientific at United States of America

Chemical Reagent	Description
Sodium carbonate	Spectrum, Chemical MFG Corp. at Gardena, California
Sodium phosphate	Sigma – Aldrich, Merck kGaA at Germany
Cetyltrimethyl ammonium Bromide (CTAB)	Loba Chemie Laboratory – India
Folin-Ciocalteau reagent	Lab. Chem. At Yachay Tech
Hydrogen Peroxide	Fisher Scientific at United States of America
Phosphate Buffer	Fisher Scientific at United States of America
Sea and	Merck kGaA at Germany
Silica	Sorbtech Rocket at United States of America
Propylene glycol	Loba Chemie Laboratory at India
Raw material for cream preparation	
Material	**Brand**
Blueberries Extract	Ecuarandano – Blueberry del Ecuador
Coconut Oil	Coco freeze – Ecopacific at Ecuador. Karay – Natural Foods at Ecuador. Weir – Labfarmaweir at Guayaquil – Ecuador.
Glycerin	Weir – Labfarmaweir at Guayaquil – Ecuador
Lecithin	Mason Natural – Mason Vitamins at United States of America

Potassium ferricyanide	Fisher Scientific at India		Lignin	Extracted from rose stems – Lab. Chem. At Yachay Tech
Sodium acetate	Fisher Scientific at United States of America			

Table 17: Laboratory materials.

Beakers: 25, 100 and 500 mL		Filter paper		Round flask
Buchner funnel		Micropipettes: 10 and 1000 μL		Spatulas
Centrifuge tubes		Mixer handheld stir		Stir bars
Chromatographic column		Plastic containers		Thermometer
Eppendorf tubes		PTFE membrane filter: 0.22μm		Volumetric Pipettes: 1, 5 and 10 mL
Extraction funnel		Quartz cuvettes		Universal laboratory support
Glass petri dish		Sample tubes for centrifuge: 13mL, 50mL		pH measuring paper

Table 18: Equipment required for the development of the project.

Equipment	Brand/ Description
Rotary evaporator	Buchi – R210
Drying stove	Pol-Eko Aparatura
Analytical Balance	COBOS precision: TX-3202L and HR-150A
Hot/Magnetic & Stirrer Plate	Mtops
Vacuum System Filtration	
Coffee grinder	Daewoo
Ultraviolet-Visible Spectrometer	Perkin Elmer / Lamba 1050
Infrared Spectrophotometer	Perkin Elmer / Version 10.4.00
UVA / UVB digital meter	General Tools UV513AB, 280-400nm
Digital Moisture Monitor for skin	SK-IV
Cellphone camera	Iphone 7 plus 12 megapixles f/1.8 2 megapixels (wide), 1/3", PDAF, OIS
Ultraviolet Lamp	Vilber Lourmat, N° 17100080
pH meter	Mettler Toledo
Digital conductivity meter	APERA USA, EC60 Premium
Centrifuge	Thermo Fisher Scientific SORVALL Legend XTR
Optical Microscope	Leica

Annex 3.

Table 19: Value of the digital moisture monitor for skin - SK-IV.

Seasons	Parts	Hydropenic (%)	Normal (%)	Excellent (%)
Spring & Autumn	Face	0 ~ 35	35 ~ 55	55 ~ 100
	Forehead	0 ~ 35	35 ~ 55	55 ~ 100
	Ocular region	0 ~ 45	45 ~ 55	55 ~ 100
	Arm	0 ~ 40	40 ~ 55	55 ~ 100
	Opisthenar (back of the hand)	0 ~ 35	35 ~ 55	55 ~ 100
	Palm of the hand	0 ~ 45	45 ~ 65	65 ~ 100
Summer	Face	0 ~ 40	40 ~ 60	60 ~ 100
	Forehead	0 ~ 40	40 ~ 60	60 ~ 100
	Ocular region	0 ~ 50	50 ~ 60	60 ~ 100
	Arm	0 ~ 45	45 ~ 55	55 ~ 100
	Opisthenar	0 ~ 40	40 ~ 60	60 ~ 100
	Palm of the hand	0 ~ 50	50 ~ 65	65 ~ 100
Winter	Face	0 ~ 30	30 ~ 50	50 ~ 100
	Forehead	0 ~ 30	30 ~ 50	50 ~ 100
	Ocular region	0 ~ 40	40 ~ 50	50 ~ 100
	Arm	0 ~ 35	35 ~ 45	45 ~ 100
	Opisthenar	0 ~ 30	30 ~ 50	55 ~ 100
	Palm of the hand	0 ~ 40	40 ~ 55	55 ~ 100