FORMULATION and EVALUATION of a COSMETIC MULTIPLE EMULSION SYSTEM CONTAINING MACADAMIA NUT OIL and TWO ANTIAGING AGENTS

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THESIS OF DOCTORATE

FORMULATION and EVALUATION of a COSMETIC MULTIPLE EMULSION SYSTEM CONTAINING MACADAMIA NUT OIL and TWO ANTIAGING AGENTS

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Naveed AKHTAR'ın DOKTORA TEZİ olarak hazırladığı "FORMULATION AND EVALUATION OF A COSMETIC MULTIPLE EMULSION SYSTEM CONTAINING MACADAMIA NUT OIL AND TWO ANTIAGING AGENTS" başlıklı bu çalışma, Jürimizce Lisansüstü öğretim yönetmeliğinin ilgili maddeleri uyarınca değerlendirilerek kabul edilmiştir.

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ABSTRACT

The purpose of the study was to prepare a stable multiple emulsion containing two skin antiaging agents using a natural oil. Vitamin C, which is a very unstable ingredient and is decomposed in the presence of oxygen, was entrapped in the inner aqueous phase of w/o/w multiple emulsion. In this way, it can produce slow release and the effect of vitamin C can be increased as it has been protected from the external environment by entrapping it in the internal phase. The other ingredient which is a product of wheat proteins was also used as an antiaging agent. Both of the ingredients increase the synthesis of collagen fibers in the dermis. Therefore, a synergystic effect can be produced by using the two ingredients in one formulation.

Multiple emulsions contain at least three phases. The material entrapped in the internal phase is released slowly and thus its effects can be prolonged. The other benefit of multiple emulsions is the protection of the material entrapped in the internal phase. In this study, multiple emulsions were prepared by the two step method because this method is more reliable and can be controlled as compared to other methods. Base containing no active material and a stable formulation, containing vitamin C in the internal aqueous phase and wheat proteins in the oily phase, were prepared. The oil used was macadamia nut oil since it contains a high quantity of palmitoleic acid which is the natural ingredient of the young skin.

Base as well as the formulation were stored at different accelerated conditions for six months to predict the stabilities of these emulsions. Both of the emulsions were found to be stable at all the different conditions. No phase separation was seen in any of the emulsions. Different tests were performed to confirm the stability of these emulsions.

Both of the emulsions were applied to the cheeks of human volunteers for four weeks. Different parameters of the skin were monitored every week to see any effect produced by these emulsions. The data obtained was evaluated statistically.

It was found that the active formulation increased the moisture of the skin as showed by statistical tests but there were no significant variation in other parameters like skin sebum, pH, elasticity, melanin and erythema.

Key Words: Multiple emulsion, Vitamin C, Wheat proteins, Physico-chemical properties, Dermatological evaluation.

ÖZET

Bu çalışmanın amacı doğal bir yağ ve iki cilt yaşlanmasını önleyici ajan kullanarak kararlı bir çoklu emülsiyon hazırlamaktır. Kararlı olmayan ve oksijen varlığında çabuk bozulan Vitamin C, S/Y/S çoklu emülsiyonun en iç sulu fazına konulmuştur. Bu şekilde, Vitamin C'nin yavaş salımı sağlanabilir ve en iç faza konulduğundan, çevresel faktörlerden etkilenmesi engellenerek etkisi arttırılabilir. Diğer etkin madde olan buğday proteini de, cilt yaşlanmasını önlemek amacıyla kullanılmaktadır. Bu iki etkin madde, dermisteki kolajen liflerin sentezini arttırır. Bu formülasyonda, iki etkin maddenin birlikte kullanılmasının amacı, sinerjik etkinin sağlanabilecek olmasındandır.

Çoklu emülsiyonlar en az üç faz içerir. Çoklu emülsiyonlarda en iç faza konulan madde yavaş salım gösterir ve böylece etkisi uzatılabilir. Çoklu emülsiyonun bir diğer yararı, en iç faza konulan maddeleri dış etkilerden korumasıdır. Bu çalışmala, çoklu emülsiyonlar iki basamaklı olarak hazırlanmıştır çünkü bu yöntem diğer yöntemlere göre daha güvenli ve kontrol edilebilirdir. Baz olarak hazırlanan formülasyonda etkin madde kullanılmamıştır. Etkin madde kullanılan formülasyonda, vitamin C en iç sulu faza, buğday proteini ise yağlı faza konmuştur. Yağ olarak yüksek oranda palmitoleik asit içeren Macadamia fındık yağı kullanılmıştır. Bu asit genç ciltte doğal olarak bulunan bir maddedir.

Baz ve etkin madde içeren bu iki formülasyon, farklı hızlandırılmış koşullarda altı ay süresince saklanmıştır. Her iki formülasyonunda, farklı koşullarda altı ay süresince kararlı kaldığı belirlenmiştir. Hiçbir formülasyonda faz ayrımı gözlenmemiştir. Bu formülasyonların kararlılığının değerlendirmesi için değişik testler yapılmıştır.

Bu iki formülasyon, 4 hafta süresince gönüllülerin yanaklarına uygulanmıştır. Emülsiyonların etkisini görmek için cildin değişik parametreleri her hafta ölçülmüştür. Elde edilen veriler istatistiksel olarak değerlendirilmiştir.

İstatistiksel testlerin sonucunda, etken formülasyon ile cilt neminde artış gözlenmiştir. Diğer parametreler olan cilt sebumu, pH'sı, esnekliği, melanin'i ve eritemde önemli bir fark gözlenmemiştir.

Anahtar Kelimeler: Çoklu Emülsiyon, Vitamin C, Buğday proteinleri, Fiziko-kimyasal özellikleri, Dermatolojik değerlendirme.

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Naveed Akhtar

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ABBREVIATIONS

UV	Ultraviolet
SOD	Superoxide Dismutase
CAT	Catalase
ME	Multiple Emulsion
RH	Relative Humidity
MED	Minimum Erythema Dose
ROS	Reactive Oxygen Species
AHA	Alpha Hydroxy Acid
LDL	Low Density Lipoproteins
LPO	Lipid Peroxidation

1. INTRODUCTION AND AIMS

Multiple emulsions are defined as emulsions in which both types of emulsions exist simultaneously (1). They combine the properties of both w/o and o/w emulsions. In addition, they have the potential advantage of prolonged release of drug, incorporation of incompatible materials and protection of active substances as they are dispersed in the internal phase (2 , 3). The preparation of multiple emulsions with natural oils is a challenging work due to the stability problems. In this study, macadamia nut oil which is a natural oil has been used. This oil has been preferred because of its cosmetics benefits for the skin. It contains proteins, fats, carbohydrates, vitamins and minerals (4, 5). Two active agents were incorporated into two different phases of the multiple emulsion.

The role of vitamin C, as an antioxidant and in the protection of skin against the deleterious effects of UVB light, has been recognized by many workers (6, 7, 8). In addition to this, vitamin C also improves the synthesis of collagens increasing the suppleness of the skin (9). This vitamin has been incorporated into the internal phase of the multiple emulsions prepared, in this study.

Proteins from wheat are being used in cosmetic products as antiaging agents (10). One such protein condensed with palmitic acid is also claimed to be antiaging (11). This protein is claimed to increase the synthesis of collagen at low concentrations and acts as moisturizer at high concentrations. This material was used in our study in the oil phase of multiple emulsion.

After the preparation of the multiple emulsions, accelerated stability studies were performed for six months by keeping the samples of the formulations, with and without the active agents, at different conditions. During this period, globule size, changes in pH, changes in viscosity and any physical change in the multiple emulsions were periodically studied in the samples kept at different conditions. One active formulation and the emulsion with no active ingredient were applied to the skin of human volunteers for four weeks. Dermatological tests including tests for irritation, moisturization, sebum and melanin content, pH and elasticity of skin were performed every week to see any effect of active ingredients. These two formulations were compared with regard to

their effects on the skin. Statistical tests were applied to evaluate the data obtained.

2. LITERATURE SURVEY

2.1. SKIN

Skin covers the entire body and protects the body from various types of external stimuli and damage as well as from moisture loss. The surface area of the skin of an adult person is about 1.6 m^2 (12). The thickness of skin varies with age, sex and location. Generally, the skin of men is thicker than that of women. However, women have a thicker subcutaneous fat layer. The skin of an average woman weighs about 3 kg, while that of an average man is 5 kg (13). In general, the skin of the eyelids is the thinnest and the sole of the foot is the thickest.

The skin is divided into three layers called the epidermis, dermis and the subcutaneous tissue. Various appendages such as hair, nails, and glands, i.e. sweat and sebaceous glands are also found in the skin. Sweat glands are distributed all over the skin, opening at tiny pores on the surface; in certain areas, such as the armpit, they are particularly prominent. Skin furrows are mostly obvious over the joints, where they correspond with folds in the deeper layer of the skin caused by joint movements. There are also skin lines which are normally invisible. The lines on the palms of the hand and the fingers are fine but very distinct, making a series of parallel curves and forming patterns which are unique to each individual.

2.1.1. Layers of Skin

2.1.1.1. Epidermis

The outermost layer of the skin is called the epidermis. It is translucent. It does not contain any blood vessels. At the bottom of epidermis is a very thin membrane, called the "basement membrane" which attaches the epidermis firmly, though not rigidly, to the layer below, i.e. dermis (14). All the cells in the epidermis originate from a single layer of basal cells, called the "basal layer", which is placed on the basement membrane. The daughter cells produced by this basal layer gradually move upwards, lose their central nucleus and start to produce skin proteins called "keratins" and fats called "lipids". They are known as keratinocytes. As the daughter cells move upwards, their shapes flatten, and they become joined by spiny structures to make another layer called "the spiny layer" (14). The cells of the spiny layer secrete lipids called "sphingolipids" which play an important function in the retention of moisture in the skin. As the cells migrate

further upwards they develop granules. This is called the "granular layer". In the upper cells of this layer, these granules discharge and fill up the spaces between the cells with lipids. As the cells rise into the top layer of the epidermis- *Stratum corneum* sometimes called the "horny layer"- take the form of flatten discs, tightly packed together. These cells are called "corneocytes" which are dead cells. The number of layers of cells in the *Stratum corneum* vary depending on the location. The cells of this layer are continually replaced by new cells and this process is called "desquamation". Artificial removal of the outer layer by cosmetic products is called exfoliation. *Stratum corneum* helps to retain moisture within the skin, and protects the body from harsh effects of the environment (14). The natural moisture flows from the deeper layers up to the top of the skin which is defined as the transepidermal water loss (TEWL). Under normal conditions, maximum 15 % of the *Stratum corneum* consists of water which decreases to 10 % when the skin is dry (14). The spaces between the cells in epidermis are filled with lipids which contain triglycerides, diglycerides, fatty acids, squalene, wax esters, cholesterol and cholesterol esters. These lipids help to regulate natural water loss. The epidermis also contains natural enzymes.

Melanocytes are the cells which are found in the basal layer of the epidermis, and release melanin- a special pigment responsible for the colors of skin and hair. The pigment is made of small structures called melanosomes. There are two forms of melanin: eumelanin which produces black and brown skin color and phaeomelanin which produces lighter skin with red hair (15). Orange yellow color of the skin is due to the presence of carotenoids in *Stratum corneum* and the subcutaneous fat layer (15).

2.1.1.2. Dermis

Beneath the epidermis, lies a much thicker layer, the dermis. Dermis is composed largely of the protein collagen. Most of the collagen is organised in bundles running horizontally through the dermis which are burried in ground substance (9). The collagen bundles are held together by elastic fibers which are made of proteins called elastin. Both collagen and elastin fibers are made of cells called fibroblasts. Special substances in the ground substance, called glycoproteins, can hold large amounts of water (15). Hyaluronic acid is another

important compound which is a part of the tissue that surrounds the collagen and elastin and which can hold and bind water hundreds of times its weight. As we get older, the amount of hyaluronic acid produced in the skin naturally decreases and therefore the skin loses its suppleness.

2.1.1.3. Subcutaneous Fat Layer

This layer consists of cells called "adipose cells" containing fatty deposits (12). It contains large nerves and blood vessels. This fatty layer acts as an insulator to conserve body heat. Fat deposition increases by the increase in age due to the decrease in body metabolism.

2.1.2. Special Skin Structures

2.1.2.1. The Sebaceous Glands

Sebaceous glands are part of the small structures, i.e. hair follicles which generate hair. These glands produce sebum which is a mixture of waxes and fats (15). Sebaceous glands are found in every part of the body except the palms and soles. Sebum is secreted through the sebaceous duct into the hair follicle. It forms a mixture with the aqueous secretion of sweat which is then distributed over the skin. Sebum is slightly acidic, has a pH value between 4.2 and 5.6 (15). In both sexes, the sebaceous glands are strongly influenced by male hormones.

2.1.2.2. Sweat Glands

Sweat glands are found in almost every part of the skin. There are two types of sweat glands, ecrine glands and apocrine glands. Ecrine glands produce sweat which is a mixture of water and salts (12). Sweat plays an important role in regulating the temperature of the body. They are found everywhere on the skin except the lips and *glans penis*. Apocrine glands are formed of the same structure as the hair follicle and sebaceous glands. They produce a highly individual sexual scent, the production of which is dependent on the presence of sex hormones (15). They are found particularly in the armpit and the genital areas.

2.1.3.Functions of Skin

2.1.3.1. Functions of the Epidermis

2.1.3.1.1. Protection From the Environment

Ultraviolet radiation is damaging for the skin. This radiation is reflected by the *Stratum corneum*, partly absorbed by melanin in the epidermal cells, and some

is scattered within the skin. This scattered radiation creates high energy particles which are called free radicals. Free radicals are very reactive and attack the constituents of the skin (12). In this way, ultraviolet radiation (UVR) leads to damages in the skin.

2.1.3.1.2. Prevention of Water Loss From The Body

Body loses water by constant evaporation through the skin which is known as transepidermal water loss (TEWL) (15). Prevention of this water loss is important both for the skin and for the body. Water makes 70-75 % of the weight of the basal layer, but only 10-15 % of the *Stratum corneum*. *Stratum corneum* is an important barrier to the moisture loss. If the water content of *Stratum corneum* falls below 10 %, it becomes dry, less flexible and prone to damage, breakdown and infections (14).

2.1.3.1.3. Prevention of Infections

The natural layer of oil-in-water emulsion (sebum and sweat) on the skin is the first barrier against invasion by microorganisms such as bacteria, fungi and yeasts. *Stratum corneum* provides the next line of defense. White blood cells in the skin destroy bacteria invading the epidermis. The epidermis also contains special defense cells called *Langerhans cells* (16). These cells mop up invading foreign substances.

2.1.3.1.4. Absorption

A variety of substances are absorbed from the skin into the body. There are two absorption paths, one through the epidermis, and one through the sebaceous glands of the hair follicles (14). Steroids such as female hormones, male hormones and adrenocortico-steroids as well as fat soluble materials such as vitamins A, D, E and K are absorbed through the skin but water soluble materials are not easily absorbed due to the barrier formed by the horny layer.

2.1.3.1.5. Synthesis

The skin synthesizes vitamin D through the action of UV light on vitamin D precursors in the skin (12).

2.1.3.2. Functions of the Dermis

The functions of dermis include:

- mechanical protection of the body from bumps and knocks,

- providing oxygen and nutrients through the blood to the living cells of the epidermis,
- removal of waste products of metabolism from the epidermis,
- contribution to the skin color,
- regulation of the body temperature through the control of blood flow and respiration
- skin sensation of touch, pain, heat and cold.

2.1.4. Aging of Skin

Skin is an organ in which changes due to aging are easily observed. The degree of changes varies greatly between individuals and also depends on the region of the body. A number of changes occur which detract from the beauty of the skin. These are called natural or intrinsic aging changes. These changes are shown in **Table 2.1.** (17).

Table 2.1. Changes in the Skin Due to Aging

. Increased wrinkles
. Increased looseness
. Reduced gloss, luster, smoothness
. Reduced elasticity
. Coarsening of skin texture and random furrows
. Pigmented spots, depigmented spots in some parts of the body
. Yellowish skin
. Thinning of scalp hair and loss of vitality
. Reduced scalp and body hair
. Increased gray hair
. Lengthening of eyebrow and ear hair
. Coarsening, muddying and bending of nails

2.1.4.1. Aging Signs of the Skin

The face and neck as well as the back of the hands are frequently exposed to sunlight and become rough and deeply lined. Skin which is continuously exposed to intense sunlight over long periods shows characteristic changes. Aging signs caused by UV rays are called photoaging (16, 18, 19). Skin of an elderly person whose regions of the body is unexposed to sunlight, such as the stomach and lower back, differ in their internal structures from the skin exposed to sun in the same person (20). Generally, in intrinsic aging, reduction in many functions and atrophic changes occur in the skin such as the reduction in cellular activity

and skin thinning (12). Conversely, photoaged skin is thickened, and there are various symptoms called "elastosis" which display the presence of massive quantities of thickened, tangled, degraded elastic fibers. **Tables 2.2. and 2.3.** show the characteristic changes in both cases (21). Both photoaging and intrinsic ageing occur in facial skin, but the degree of aging differs from individual to individual because while photoaging is affected by the life-style such as the amount of time exposed to sunlight and type of daily skin care, intrinsic aging is affected by genetic factors and other internal factors. **Table 2.2 and 2.3** also show the internal changes in intrinsic and photoaged skin.

Table 2.2. Epidermal Changes in Aged Skin (22).

Item	**Photoaged Skin**	**Intrinsic Aged Skin**
Epidermal thickness	Thick epidermis	Thin epidermis
Epidermal cells (Keratinocytes)	.Non-uniform cells .Randomly distributed cells .Loss in polarity .Frequent enlargement .Diversified melanosomes	.Uniform cells .Defined cells distribution .Polarity maintained .Usually atrophied .Uniformaly distributed melanosomes
Stratum corneum	.Increased number of cell layers .Diversified form and size of corneocytes	.Normal cell layers .Uniform corneocyte size
Melanocytes Langerhans cells	.Cell number increased .Diversified cells .Increased melanosome production .Marked reduction in cell number	.Cell number reduction .Uniform cells .Poor melanosome production .Slight reduction in cell number

Table 2.3. Dermal Changes in Aged Skin (23).

Item	Photoaged Skin	Intrinsic-Aged Skin
Glycosaminoglycans	.Marked increase	.Slight decrease
Elastic Tissues	.Tremendously increased .Degenerated into amorphous mass	.Increased but almost normal
Collagen	.Marked decrease of bundles and fibers	.Bundles thick and disoriented
Reticular Dermis Fibroblasts Mast cells Inflammatory cells	.Thickened .Increased and hyperactive .Increased .Inflammatory cell penetration	.Thinner .Decreased and inactive .Decreased .No inflammatory cells
Papillary dermis	.Grenz zone of new collagen	.Non grenz zone of new collagen
Capillary vessels	.Great loss in small vessels .Abnormal vessels .Telangiectatic	.Moderate loss . Normal .Non-telangiectatic
Lymphatics	.Practically absent	.Moderate loss

2.1.4.2. External Changes in Aged Skin

2.1.4.2.1. Wrinkles

Wrinkles occur in almost all parts of the body especially in the forehead, around the eyes, between the eyes, around the mouth, and on the nape of the neck, elbows, armpit, feet and hands. They usually start appearing around the age of 30 and increase in number, depth and area with aging. There are various types of wrinkles and several classifications. Kligman (24) has classified wrinkles as:

- Linear wrinkles commonly called "crows feet" around the outer corner of the eyes,
- Glyphic wrinkles which crisscross triangular or rectangular wrinkles commonly seen on the cheeks and neck,
- Crinkling, i.e. fine wrinkles commonly seen on the regions unexposed to sun of elderly people.

Linear and glyphic wrinkles reflect photoaging changes and crinkling reflects intrinsic changes. The wrinkle formations are caused by various internal and external factors. UV light is known to be one cause, but there are also other causes such as environmental stresses on the skin including dryness and physical and chemical stress. Wrinkles are thought to be formed by the loss of tension and elasticity through interactions between reduced water content of the *Stratum corneum*, atrophy of the epidermis, changes in the amount and quality of dermal collagen and elastic fibers, change in the three dimensional structure of the dermis and other changes resulting from external and internal factors (25).

2.1.4.2.2. Sagging

Sagging of the skin starts around 40 years of age and is most common in the chin, eyelids, cheeks and sides of the stomach. The causes of the sagging are the same as the wrinkles. There is reduced elasticity of the dermis and reduced support by the subcutaneous adipose tissues. The strength of muscles supporting the skin is also reduced (25).

2.1.4.2.3. Surface Configuration

With aging, skin surface relief, which is formed by furrows and ridges, becomes more shallow and also less precise. The direction of the skin furrows becomes irregular, and skin pores tend to become larger (26).

2.1.4.2.4. Pigmentation and Skin Color Changes

The pigmented spots of skin generally increase with age (27, 28). Skin tends to become darker (28). The changes are thought to be related to reduced transparency caused by increased pigmentation, reduced secretion of sebum and thickening, and reduced water content of the horny layer due to aging.

2.1.4.3. Changes in Skin Functions

2.1.4.3.1. *Stratum Corneum*

The most important function of *Stratum corneum* is the retention of water which is decreased with aging. Reduction in the skin surface lipids and perspiration are the factors in the drying of the skin in elderly people (29).

2.1.4.3.2. Epidermis

Proliferation of epidermal cells is reduced in aging. Consequently, metabolism in epidermis is also reduced (15). The size and the surface area of the corneocytes increase with the increase in aging.

2.1.4.3.3. Dermis

The proliferative activity of fibroblasts in the dermis decreases with aging. Production of collagen, elastin and glycosaminoglycans by the fibroblasts also decrease with age (30). As the turnover rates of collagen and other structural proteins are very slow, various degenerative changes such as cross-links occur on these components which result in the reduction of skin elasticity (30).

2.1.4.3.4. Subcutaneous Adipose Tissues

Aging causes reduction in subcutaneous adipose tissues and the skin tends to become yellowish as a result of increased cholesterol levels (15). The reduction in subcutaneous adipose tissue reduces the ability to withstand physical shocks and is also thought to be a cause of wrinkles (12).

2.1.4.3.5. Amount of Skin Lipids

The amount of sebum declines with age. This is observed more clearly in women than men. The degree of change with age differs according to the region of the face involved (31).

2.1.4.3.6. Skin Blood Flow

The blood flow depends on the body region involved. However, generally, there is reduction in flow with aging and reduction in the ability to withstand cold and UV irradiation (32).

2.1.5. UV Light and Skin

Ultraviolet light is at wavelengths shorter than the visible light. It is divided into three regions: UVC (200-280 nm), UVB (280-320 nm) and UVA (320-400 nm) (12). UVC is filtered by ozone and does not reach the earth. The shortest UV wavelength reaching the skin is in the range of 290-320 nm and the energy of UVB component is about 1/10 or 1/20 of the UVA content (12). The

strength and the amount of UV light vary greatly depending on the geographic position, season and time of day. Usually, the UV radiation peaks at 12:00 A.M., half of the daily amount of UV light is received between 10:00 A.M. and 14:00 P.M (12).

The skin has a natural defense mechanism against the UV light. UV radiation is scattered and absorbed by the structure and structural materials of the skin to attenuate the amount reaching the deeper layers (12). Among these, melanin produced by melanocytes in the basal layer shows a very effective UV protection effect. The amount of UV light which penetrates the skin as a result of these factors varies according to the wavelength. Longer wavelengths penetrate into deeper layers of the skin (33).

2.1.5.1. Response of Skin to UV Light

Skin shows two types of responses to UV light: 1) Acute response;
2) Chronic response.

2.1.5.1.1. Acute Response

The skin becomes darker immediately after exposure to UV light. This darkening is the result of the oxidation of existing melanin pigment (12). This response is initiated by UVA light. Several hours after exposure to UV light, skin begins to become red. If exposed to very high amounts of UV light, blisters develop and the skin feels burnt. UV light in the 290-320- nm, i.e. UVB, is more effective in producing sunburn (12). The cells damaged by UV light produce an inflammatory mediator, dilating the capillaries and resulting in the appearance of sunburn. Anti-inflammatory drugs like aspirin can inhibit erythema occuring several hours after UV exposure (12).

Approximately three days after exposure to UV light, the skin gradually becomes darker. This suntan is a result of acceleration in the production of melanin and with its movement into the keratinocytes. Suntanned skin gradually returns to its original color only after several months.

The minimum erythema dose (MED) is the minimal amount of UV light required to cause redness when a person is exposed to UV light (15). Individuals with high sensitivity have a low MED because only a small amount of UV light is required to cause skin redness. The erythema response and darkening also differ

among the individuals. Some people are oversensitive to UVA while others are more sensitive to UVB. These responses are called "photosensitivity" and may be classified as phototoxic response, photoallergy response and photohypersensitivity response (12). The phototoxic response can occur in any person applying material to the skin and then exposing themselves to sunlight. The photoallergy response is related to the immune system, generally occurs only in photo-sensitized individuals. Photosensitivity occurs in xeroderma pigmentosum, photoatopia, porphyria and other viral conditions (12).

2.1.5.1.2. Chronic Response

The skin which is dark, feels rough to the touch, and is deeply wrinkled. The nape of the neck, which is constantly exposed to UV light, has characteristic diamond-shaped wrinkles (18). If this condition worsens, skin cancer may result (18, 34). Since these types of changes are different from natural aging these are called "photoaging". When the photoaging changes are examined histologically, epidermal thickening and overdeveloped melanocytes are observed (35). Photoaged skin has an abnormal increase in the amount of elastic fibers and the fine dermal blood capillaries are dilated (35). The immune system is also affected by chronic exposure to UV light (35).

2.1.6. Smoking And Skin

Smokers look older than the non-smokers of the same age. In a study by Christine et al (36), the concentration of mRNA for matrix metalloproteinase 1 (MMP-1) in the buttock skins of smokers and non-smokers were compared. MMP-1 degrades collagen which accounts for at least 70 % of the dry weight of the dermis. It was reported that significantly more MMP-1 mRNA was present in the skin of smokers than non-smokers. It was suggested that smoking-induced MMP-1 could be important in skin ageing effects of tobacco smoking (37). These findings confirm and validate similar in-vitro results in dermal fibroblasts (38).

2.1.7. Theory of Aging

Among the various theories describing the aging process, the free radical theory of aging first suggested by Harmann (39) has gained popularity. This is the general theory which presents explanations for many phenomena and events observed along the aging process.

2.1.7.1. Reactive Oxygen Species (ROS)

The role of ROS in skin damage and skin aging is commonly associated with the free radical theory of aging (25). The generation of free radicals triggers cross linking and other reactions which lead to the types of damage or deterioration commonly associated with aging (40). Free radical formation occurs on and in the epidermis. Internal sources of free radicals are from the respiratory chain, prostaglandin synthesis, the cytochrome p-450 system and the oxidative burst during phagocytosis (41). These enzymetically generated free radicals contribute extensively to internal aging phenomenon. On the skin, however, free radical generation from ionizing and less energetic radiations appears to be the major contributor to aging phenomenon. Phenomena in which ROS are not involved also exist in the response of skin to external radiation, e.g. the isomerization of urocanic acid is a response to incident UV radiation, but does not involve oxygen and probably does not contribute to the loading of ROS in the skin. The ROS involved in the reactions are singlet oxygen ($^1O_2$), the superoxide radicals ($HO_2^{\cdot}$ or $^{-}O_2^{\cdot}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$). The reactions of ROS with skin components and the resulting adverse effects on skin are of cosmetic interest.

2.1.7.1.1. Skin Lipid Damage By ROS

UV radiation on human skin results in transit reduction of superoxide dismutase (SOD) (42). On the other hand, the amount of catalase (CAT) almost doubles following irradiations *in vivo*. This type of exposure also has effects on glutathione transferase (GSH-TF) but not on glutathione peroxidase (GSH-PX). The post irradiation peak in the level of conjugated double bond reflects free radical damage to natural skin lipids.

UVB light, in the presence of oxygen, adversely affects the body's natural reductive defenses (SOD and CAT) in the skin and leads to the formation of ROS in the skin (42). These conclusions are fully supported by the data of *in vitro* UVA radiation on keratinocyte cultures (43). The natural protectants against ROS in the skin are superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, tocopherols, ubiquinones, ascorbic acid and dehydro-ascorbic acid. Witt et al (44) and others (45) demonstrated the presence of hydroperoxides in

UV irradiated hairless mouse skin and suggested that skin damage could be prevented by "bolstering antioxidant defenses".

Loss of natural antioxidants present in skin as a result of UV irradiation may trigger peroxidative damage- under the influence of ROS- to unsaturated lipids (46). This problem has been studied repeatedly and investigators have concluded that topical application of known antioxidants can reduce these adverse effects (47). Bissett et al (48) studied the ability of various antioxidant species to interfere with the destructive effects of different ROS. These investigators examined not only the *in vitro* human skin penetration of commonly employed antioxidants but also their ability to scavenge superoxide *in vitro*. The data suggests that ascorbic acid penetrates the skin poorly. Superoxide scavenging is quite selectively performed by ascorbic acid and tocopheryl acetate. It is evident that mode of action of "antioxidants" against ROS is quite specific.

In hairless mice, UV irradiation causes reduction in all antioxidant defense systems. The loss in the enzymes, tocopherol, ubiquinols, and ascorbic acid are significantly higher in the epidermis than in the dermis (49). This suggests that the primary damage to skin during insolation occurs in the topmost layer.

The observation of conjugated double bonds in irradiated skin *in vivo* is probably due to the oxidative damage of linoleic acid (41). This acid is an important component of ceramide-1 and a key constituent of essential fatty acids. Lipid peroxides (ROOH), although fairly stable, are a potential source for RO· and ROO· radicals. The latter may react with proteins, such as low-density lipoproteins. Malonaldehyde and 4-hydroxynonenal react aggressively with histidine residues (50). Thus, lipid peroxidation not only damages lipid via peroxidation, but the peroxidized lipids can subsequently damage proteins.

The peroxidation of skin lipids includes reaction of squalene (51). Squalene is decomposed under ambient laboratory light conditions, more rapidly under UV light, and still more rapidly in the presence of a porphyrin (Porphyrins are photodynamic agents and generate singlet oxygen during irradiation).

Cholesterol, a natural constituent of epidermal lipids, and sphingosine, a constituent of various ceramides in *Stratum corneum*, are unsaturated and subject to oxidative attack. One of the first photooxidation products of membrane bound

cholesterol is the 5-α hydroperoxide (52). Autooxidation can produce 5,6-epoxide and especially 7- ketocholesterol (53). The formation of these degraded products may not be critical for skin structures, but at least one of the formed epoxide is known to be carcinogenic (54). Girotti et al (52) reported that catalase, SOD and phenolic antioxidants inhibit the formation of these undesirable cholesterol oxidation products.

2.1.7.1.2. Skin Protein Damage by ROS

Proteins and amino acids in the skin are also subject to oxidative degradation but the mechanism by which this occurs is not clear. Free radicals ($^{\cdot}OH$ and $^{\cdot}OOH$) are known to alter enzyme activity, probably as a result of damage to prosthetic groups such as methionine, tryptophane, histidine and cysteine (55). Even mild oxidative stress resulting from the reaction of ascorbic acid with oxygen in the presence of trace metals can damage albumin by formation of dimeric tyrosine products. This demonstrates that ROS can be formed under very mild conditions (55).

Some repair mechanisms for damaged enzymes exist (e.g. methionine sulfoxide reductase) (56). Enzyme damage seems to be due to oxidative reactions, primarily. In addition, $^{\cdot}OH$ and $RO^{\cdot}$ can cross-link proteins or rupture them. Fragmentation is the most frequent reaction. Wolff et al (56) proposed a scheme where the α-carbon of an amino acid is oxidized to yield an α-carbon peroxy radical. Reviews of the effect of free-radical-catalized oxidative modification of proteins deals with proteins as a group and the rupture of amino acid for which $^{\cdot}OH$ radicals are primarily responsible (56, 57).

Protein degradation may disrupt the activity of keratinocytes and may include fragmentation of enzymes which are specifically required in the *Stratum corneum* and the viable epidermis. One of the most damaging effects of ROS in skin appears to be collagen denaturation and cross linking (41). Pathak et al (58) irradiated solutions of soluble guinea pig skin collagen *in vitro* in the presence or absence of photosensitizers. Denaturation resulted in clouding of the solution and was materially reduced in the absence of oxygen. Singlet oxygen quenchers (β-carotene or α-tocopherol) delayed the occurance of clouding. Interference with the formation of ROS evidently reduces collagen cross linking (58).

2.2. ANTIAGING MATERIALS

2.2.1. Vitamins and Skin Aging

Laboratory and clinical studies have indicated a useful role of topically applied vitamins in combating various skin disorders, especially in preventing, retarding or arresting certain degenerative changes associated with the aging process, such as dry or scaly skin and the formation of wrinkles. In addition, the natural character of vitamins has prompted their use in creams and lotions to maintain a soft and smooth skin by "replenishing nature's moisture". Vitamins E, A, C, panthenol and their derivatives are of particular interest. These vitamins are functional, penetrate the skin, and when used at proper levels, are safe and free of side effects.

2.2.1.1 Vitamin E

This vitamin is necessary for the maintenance of normal body metabolism, for the protection of body tissues and skin from damage caused by normal body processes (59). Additionally the main claim for vitamin E in cosmetics is that it is a "natural moisturizer". Vitamin E protects the skin from UV damage and has an anti-inflammatory effect. Topical vitamin E acetate application was found to reduce sunburn response at low MED (60).

Vitamin E occurs as tocopherol of which α-form has the highest biological activity. Unesterified α-tocopherol has an antioxidant activity *in vitro* and is a physiological antioxidant *in vivo*. A relatively high proportion of topically applied vitamin E has been found in the *Stratum corneum* and in the lower viable skin layers.

2.2.1.2. Vitamin A

Vitamin A is essential not only for normal skin development, but also for growth and maintenance of bones, glands, teeth, nails and hair. Vitamin A is absorbed through the skin, helping it remain soft and plump and improves the skin's water barrier properties. The stimulatory effect of vitamin A tends to oppose changes that occur with aging. By the topical application of vitamin A, the skin is activated to produce more epidermal proteins and to form a thicker epidermis covered by a better formed keratin layer (61, 62). There is evidence that this vitamin can also alter or modulate collagen synthesis (63).

β-carotene, a precursor of vitamin A, appears to protect the skin against damage and dehydration caused by UV irradiation. In humans, vitamin A diminishes peroxidation induced by UV irradiation (64).

2.2.1.3. Panthenol

Panthenol is the biologically active alcohol analogue of pantothenic acid, a vitamin of the B-complex group which is a natural constituent of skin and hair (41). When applied topically, panthenol is converted to pantothenic acid. It is essential for the normal functioning of epithelial tissues (41). Skin's deficiencies of this vitamin include cornification, depigmentation and desquamation. Cellular regeneration is accelerated by topical application of d-panthenol. Because of its ability to penetrate into the skin and because it has a humectant character, panthenol can act as a skin moisturizer (41).

2.2.1.4. Vitamin C

Topical vitamin C is claimed to inhibit UV radiation induced damage to porcine skin (29). It functions as a biological co-factor and antioxidant (47). It has been reported to be important in treating pathologies ranging from common cold to cancer, primarily due to its antioxidant functions. (Since vitamin C is one of the active ingredients used in this study, its properties and characteristics are given in detail in chapter 2.5.)

2.2.2. Enzymes and Skin Aging

The natural sloughing process is enzymatically controlled and specific enzymes dissolve the desmosomes releasing the dead surface cells. These enzymes are produced in the dead keratinocytes. Their active sites are sulfhydryl groups, to cleave peptide bonds (65).

One field where the topical application of enzymes has been shown to have significant benefits is skin protection. These are enzymes to capture free radicals, preventing damage to skin caused by environmental pollution, bacteria, smoke, sunlight and other harmful factors.

2.2.2.1. Superoxide Dismutase (SOD)

SOD works by dismutation, a process by which reactive oxygen free radical is converted to a less reactive form.

2.2.2.2. Catalase (CAT)

Catalase is an enzyme which converts harmful hydrogen peroxide to water and oxygen molecules.

2.2.3. Alpha Hydroxy Acids (AHA) and Skin Aging

These compounds occur naturally in fruits, sugar-cane and yoghurt. These include glycolic acid, lactic acid, malic acid, tartaric acid and citric acids. Under normal conditions of purity, temperature and pressure, AHAs have their functional portion apparently free. In 70's, AHAs were used to adjust the pH of cosmetic products but later it was realized that they produce beneficial effects on skin (66). In 1974, Middleton (67) published the favourable effects of sodium lactate in the treatment of cutaneous dryness and flaking. Van Scott and Yu (68) reported the use of AHA's in skin disorders characterized by hyperkeratosis, i.e. excessive thickness of *Stratum corneum* that sloughs off corneocyte aggregates instead of separate dead cells. AHAs and α-keto acids (AKAs) reduce the thickness of hyperkeratotic *Stratum corneum* by decreasing corneocyte cohesion, in topical use (69). It has been suggested that topical AHAs might start the biosynthesis of dermal glycosaminoglycans and other intercellular ground substances which could be responsible for the eradication of fine wrinkles (69).

An immediate improvement in skin smoothness and longer term rejuvenating of the skin can be the result of successful hydroxy acid therapy (70).

2.2.4. Hormones and Skin Aging

Hormones are regarded as cosmeceuticals. Due to their contribution to the cosmetics properties of the skin, they are included in the cosmetic materials. Both estrogen and androgen receptors have been identified on dermal fibroblasts and epidermal keratinocytes (71). Histological studies have indicated a thinning of the epidermis with age and with castration. This was reversed by estrogen replacement (72). Benificial changes using topical estradiol gel has been shown to increase skin collagen content as measured by skin hydroxy proline (73). So these preparations may be regarded as cosmeceuticals.

Estrogen appears to help in the prevention of skin aging in several ways. This reproductive hormone prevents a decrease in skin collagen in postmenopausal women; topical and systemic estrogen therapy can increase the skin collagen content and therefore maintain skin thickness. Additionally,

estrogen maintains skin's moisture by increasing mucopolysacchrides and hyaluronic acid in the skin and possibly maintaining *Stratum corneum's* barrier functions.

2.2.5. Proteins and Skin Aging

Cosmetic proteins and their derivatives can be used in skin care applications to provide functional benefits such as moisturization and skin tightening. The choice of proteins, i.e. low molecular weight moisturizer or high molecular weight film former, is however important if claims and benefits are to be substantiated (74).

The functionality of cosmetic proteins is ultimately related to the structural composition and molecular weight of the protein itself. Lower-molecular-weight enzyme hydrolysates and amino acids behave primarily as moisturizer and may penetrate the *Stratum corneum* and epidermis and into the lower dermal layers (74). High-molecular-weight proteins, however, do not penetrate significantly into the epidermis; instead they remain at the surface of the *Stratum corneum* where they behave as excellent film formers and modifiers of tensile properties- effects that are perceived as skin tightening or smoothing (74).

Several of the lower molecular weight enzymes in hydrolyzed proteins are excellent moisturizers when applied as o/w emulsions and liquid detergents (10). Higher molecular weight proteins and protein copolymers, on the other hand, repeatedly demonstrate their ability to tighten the skin acting as tranparent smoothers and antiwrinkle agents (74). Proteins have the ability to:

- increase skin extensibility and level of moisturization from both leave-on and rinse-off formulations,
- provide a skin tightening effect,
- improve the ability of skin to recover from an applied deformation, thus temporarily reversing the effect of age on the skin's mechanical properties (74).

The generation of free radicals by ultraviolet light accelerates skin aging, which is known as photoaging. Cutaneous iron catalyzes the generation of free radicals. Minabu and Keiji (75) designed novel antioxidants which suppressed the iron-catalyzed free radical generation and the ensuing UV-induced damage by

mimicking the binding site of iron sequestering proteins. These antioxidants, N-(2-hydroxybenzyl) amino acids, were prepared by condensation of amino acids such as glycine and serine with salicylaldehyde and followed by a catalytic reduction. The compounds formed a 2:1 complex with iron ion. These amino acid derivatives inhibited the iron induced hydroxyl radical generation (the Fenton reaction). The compounds also suppressed UV-induced lipid peroxidation in murine dermal fibroblast homogenates. In addition, N-(2-hydroxybenzyl)-serine showed protective activity against UV-induced cytotoxicity in murine dermal fibroblasts. Desferrioxamine, a strong iron sequestering compound, was effective in inhibiting the Fenton reaction and the lipid peroxidation, but it was ineffective in protection against UV- induced cytotoxicity (75). The results suggested that UV-induced oxidative stress can be reduced by these amino acid derivatives.

Feri et al (76) developed two 3-dimensional culture systems, in which the cells develope within a porous structure, reproducing the extracellular matrix of the human dermis. The dermal equivalent model used in this study was composed of a dermal matrix made of collagen-chitosan-glycosaminoglycan populated by normal human fibroblasts which synthesized their own extracellular matrix (76). A skin equivalent model was obtained by the cell culture of normal human keratinocytes onto a dermal equivalent elevated at the air-liquid interface. Such models were used to prove antiaging activity of promising compounds. Cosmetic science has used many protein hydrolysates in order to fight against skin aging, but upto now, natural peptides were poorly studied, and their efficacy was poorly demonstrated. Eight protein hydrolysates were screened in a proliferation study in monolayered cultures giving two selected polypeptides (76). A soya derived peptide was used for an efficiency study in 3-dimensional models. In the dermal equivalent model, this peptide increased fibroblast proliferation by 40 % and led to a stimulation of collagen formation (65 %) and elastin synthesis (16 %). The effect of this soya peptide on glycosaminoglycan synthesis was also significant, with an increase of 36 % for chondroitin-4-sulfate and 68 % for hyaluronic acid. These results were confirmed using a skin equivalent model. In this method, the soya peptide increased the thickness of the epidermis (76).

2.3. MULTIPLE EMULSIONS

2.3.1. Definition.

For the first time in 1925, Seifriz (77) reported the existence of multiple-type dispersion during the phase inversion of ordinary emulsions. The term "multiple emulsions" was then recognized as the two liquid phases separated by another immiscible liquid layer.

Becher (1) described water-in-oil-in-water (w/o/w) emulsions in his book as "an emulsion in which both types of emulsions exist simultaneously". This means that an oil droplet may be suspended in an aqueous phase, which in turn encloses a water drop, thus giving a w/o/w emulsion.

Shermann (78) interpreted the oil-in-water-in-oil (o/w/o) as "a w/o emulsion containing very high volume of water showing that the globules are no longer simply water droplets. Indeed, small droplets of oil are dispersed in water droplets and the condition may even be reached where smaller water droplets are still dispersed in the small oil droplets".

Sheppard et al (79) proposed the system of subscript to clearly define the individual components and sources. The aqueous dispersed phase, w_1, of the starting w/o emulsion becomes the aqueous compartment of the w/o/w emulsion in the case of the two separated steps of emulsification **(Figure 2.1).** The traditional aqueous phase, w_2, which is mixed with the starting w/o emulsion, forms the aqueous suspending fluid of the w/o/w emulsion, while the continuous oil phase, o, of the starting w/o emulsion becomes the oil layer separating the aqueous compartment from the aqueous suspending fluid with a vesicular structure. Therefore, the notation w/o/w for this system should be $w_1/o/w_2$.

W/O/W Multiple Emulsion O/W/O Multiple Emulsion

Figure 2.1. Schematic Representation of Multiple Emulsion Types

2.3.2. Manufacture

Several processes are used for the manufacture of multiple emulsions. None is universal and each process must be modified according to each formulation.

2.3.2.1. Two-Step Method

This is the most widely used procedure (79, 80). The 1st step consists of preparing a primary emulsion. In the 2nd step, a definite amount of this primary emulsion is dispersed in an external phase containing the secondary emulsifier **(Figure 2.2.)**. Although it is a simple method, the 2nd step of the procedure includes many critical factors. Two-step method is summarized in **Figure 2.2.**

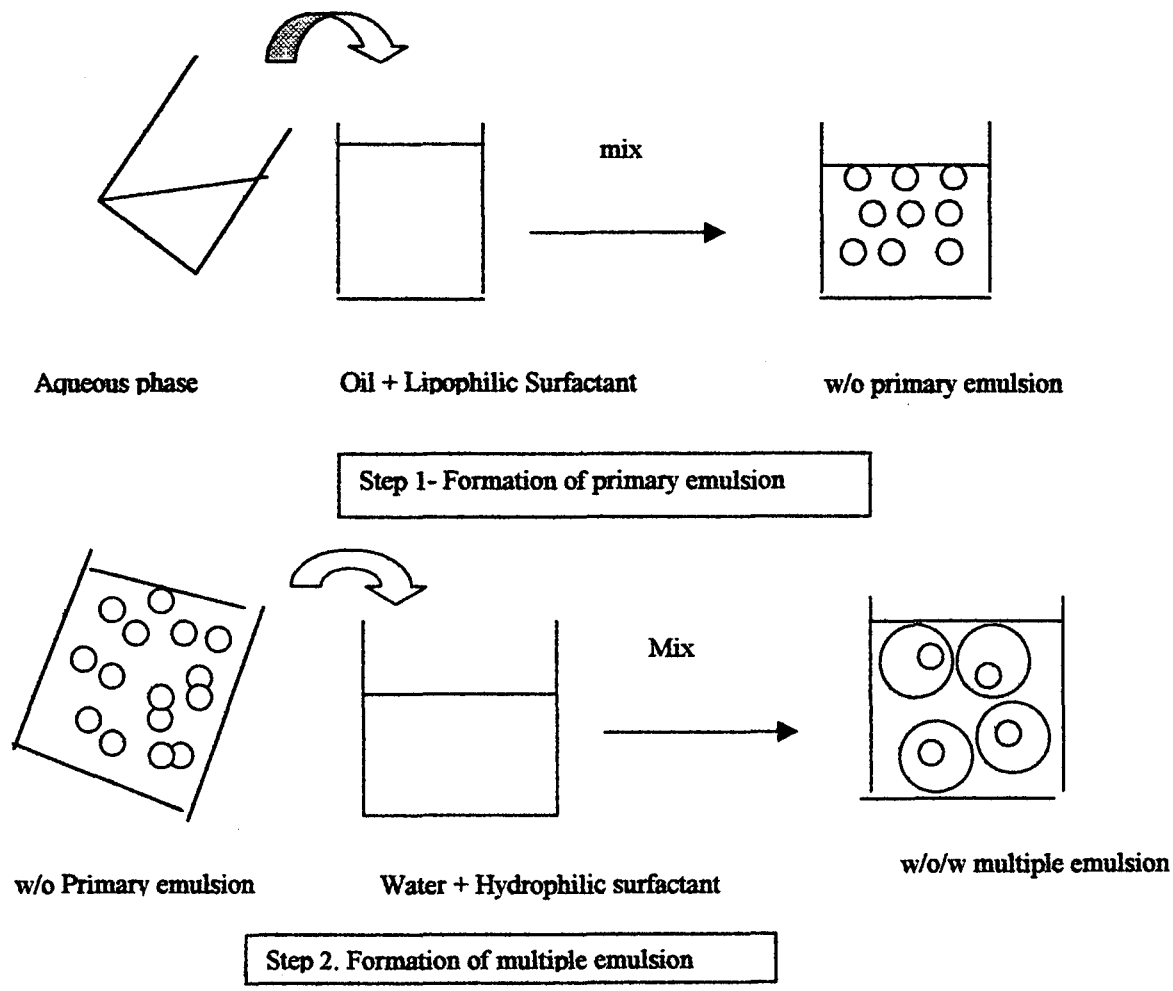


Figure 2.2. Two Step Preparation of a w/o/w Multiple Emulsion

Each parameter of the two emulsification procedure has an influence on the characteristics of the system. In the manufacture of a w/o/w multiple emulsion, the 1st emulsification step involves the requirements which are the same with all other w/o emulsions, such as a high manufacturing temperature, i.e. 70-80°C, a high agitation speed, usually above 1500 rpm, with a high powered mixer for about 20 to 35 minutes until complete cooling to ambient temperature (79).

During the 2nd manufacturing step, in order to avoid the breakdown of the oil droplets, some precautions have to be taken. The introduction of the primary emulsion into an aqueous emulsifier solution should be carried out at ambient temperature and should be slow and progressive, the manufacturing speed should be relatively low. After the complete introduction of the primary emulsion, the stirring should be continued for at least 10-30 minutes at a low speed of 200-800 rpm for the homogenization of multiple emulsion.

2.3.2.2. Phase Inversion Method

This is also a two-step emulsification process. The only difference between these two methods is that the external phase is incorporated into the primary emulsion during phase inversion method instead of incorporating the primary emulsion into the external phase during the two-step process mentioned above **(Figure 2.3.)**.

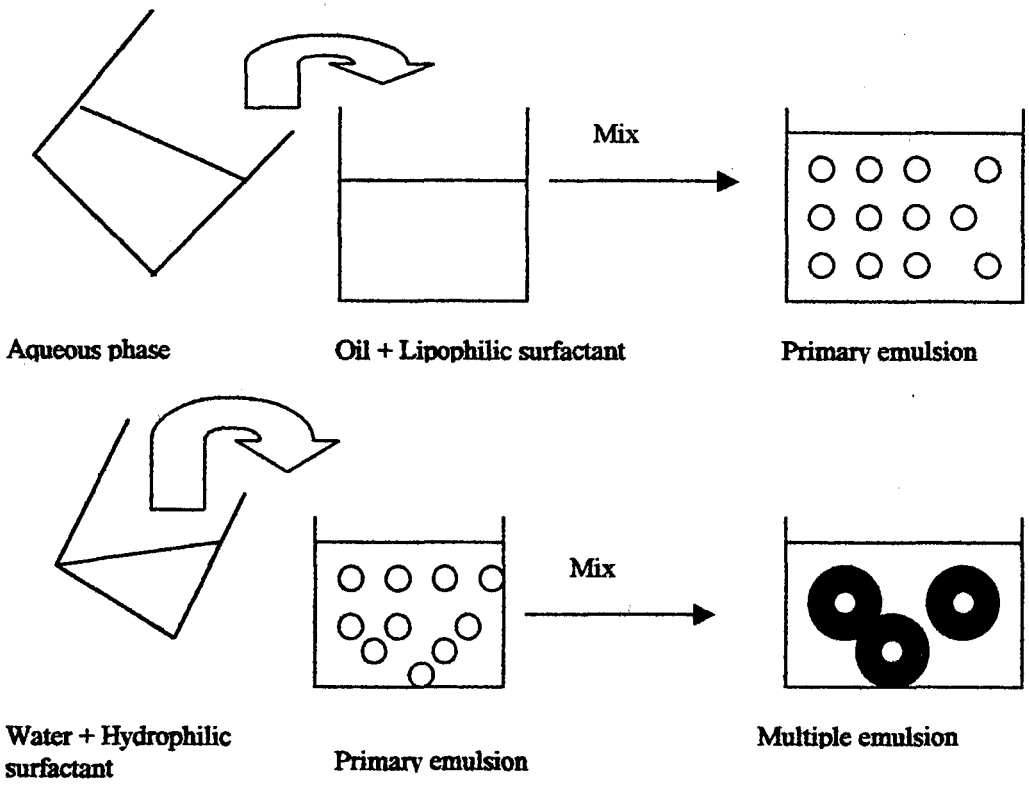


Figure 2.3. Preparation of w/o/w Multiple Emulsion by Phase Inversion Method

As can be followed in **Figures 2.2. and 2.3.**, the 1st step is the same for the two methods, i.e. introduction of water into the oily phase containing the lipophilic emulsifier. However, in the 2nd step, aqueous external phase with hydrophilic emulsifier is added to the primary emulsion slowly at room temperature, which is opposite of the two-step method. This method (Phase inversion method) is usually used for the preparation of o/w/o emulsions.

2.3.2.3. Lamellar Phase Dispersion Method

This method has been reported by Vigie (81). The method has been derived from the process employed for the production of liposomes using nonionic emulsifiers and it can only be used for the constituents which form a lamellar phase upon mixing with water. The lamellar phase is prepared with about 60% emulsifier, 10% oil and 30% demineralized water containing electrolytes by mixing at 70°C (81). The dispersion is obtained with a turbine agitator at 500 rpm for 10 minutes. Following the formation of mesophase, a given amount of water is

added at a very high agitation speed, i.e. > 1500 rpm until the formation of a w/o/w multiple emulsion.

2.3.2.4. Other Methods

Kavaliunas et al (82) described a method by mixing various phases: oil rich solution of emulsifier, aqueous micellar solution of emulsifier, and mesomorphous phase. This method is rarely used.

Pilmann et al (83) prepared a system using an inverse micellar solution and soybean oil. The system obtained was emulsified with an aqueous phase containing sodium caseinate. This method allowed the preparation of a w/o/w microemulsion.

In 1984, Makino et al (84) described a mechanical method in one step with continuous agitation. In this method, they mixed equal quantities of an aqueous phase containing a very low quantity of hydrophilic emulsifier and the oil phase containing a high proportion of lipophilic emulsifier. The equipment used was specific and allowed a constant speed of high agitation.

Higashi et al (85), in 1995, described a new method of producing w/o/w multiple emulsions by the membrane emulsification technique. This method permits the formation of monodispersed lipid microdroplets containing aqueous microdroplets to form a w/o/w system. In this method, the aqueous internal phase is mixed with an oil phase containing lipophilic emulsifier. This mixture is sonicated to form a simple w/o emulsion which is filled into the upper chamber of a special apparatus. The external aqueous phase containing the hydrophilic emulsifier is continuously injected into the lower chamber to create a continuous flow. Nitrogen gas fed into the upper chamber initiates a permeation of w/o emulsion through the controlled-pore glass membrane into the emulsifying chamber, forming a w/o/w multiple emulsion.

2.3.2.5. Factors Affecting Manufacture of Multiple Emulsions

Factors affecting the manufacture of multiple emulsions, can be classified in two groups:

- Formulation factors
- Physical factors

2.3.2.5.1. Formulation Factors

2.3.2.5.1.1. Nature of the Oil Phase

The oil phases most frequently used to form multiple emulsions are hydrocarbons, triglycerides and esters. Among hydrocarbons, liquid paraffin (86) is the most widely used. The main triglycerides used are peanut oil (87), olive oil (88), sesame oil (89), almond oil (90), maize oil (91), castor oil (92) and soybean oil (80). Among esters, isopropyl myristate (93) is widely used. Stable multiple emulsions have been produced with fatty acids (94), fatty alcohols (95) and various silicones (3), used alone or mixed with other oils. Semisynthetic oils (80) have also been used.

The nature of the oil has an influence on the characteristics of the multiple emulsion system. Therefore, physico-chemical properties of oils such as polarity, density and viscosity influence the behaviour of emulsions (94). The nature of the oil phase in w/o/w multiple emulsion is responsible for the permeability characteristics of the oil layer and, consequently, the release characteristics of the drugs or markers from the internal aqueous phase (95).

2.3.2.5.1.2. Nature of the Emulsifiers

Nonionic emulsifiers are preferred in the cosmetic industry because they have good cutaneous tolerance and compatibility. Among those nonionic emulsifiers, the most often used are those with an ester bond with saturated or unsaturated chain of fatty acid, e.g. sorbitan stearate (96), sorbitan oleate (97), ethoxylated sorbitan stearate (98) and ethoxylated sorbitan oleate (80). Non-ionic emulsifiers with an ether bond such as ethoxylated fatty alcohols are rarely used. However, polymeric emulsifiers such as poloxamers (polyoxyethylene/polyoxypropylene block copolymers) (99), Abil® (polysiloxane polyalkyl polyether copolymer) (100) and Hypermer® (modified polyesters) (101) are frequently used. For the preparation of multiple emulsions, two different kinds of emulsifiers are used. One is lipophilic and the other is hydrophilic. The HLB value of the emulsifier mixture should generally be less than 11. If this value is greater than 11, there is a risk of phase inversion (102). The HLB value of the emulsifier mixture can be calculated by Frenkel's equation (103).

$$\text{Weighed HLB} = \frac{HLB_1 . \phi\,(w/o/w) . W_1\,\% + HLB_2 . W_2\,\%}{\phi\,(w/o/w) . W_1\,\% + W_2\,\%}$$

Where, HLB_1 and HLB_2 are the HLB values of primary and secondary emulsifiers, respectively; W_1 % and W_2 % are the percentages of primary and secondary emulsifiers; and ϕ (w/o/w) is the volume fraction of the w/o emulsion in multiple emulsion.

2.3.2.5.1.3. Nature of Entrapped Substances

Substances which are added into the internal phase may be active or markers. These substances influence the characterization and stability of the systems. Electrolytes (NaCl, $MgSO_4$) (79) are often used for the purpose of:

- increasing the stability of the system by the formation of a rigid interfacial layer
- promoting an osmotic balance between the internal and external aqueous phases.

2.3.2.5.1.4. Nature of Additives

The substances which are added into any of the phases may have an influence on the characterization and stability of the system. A wide range of substances may be added into the internal aqueous phase such as proteins (117), drugs (101) and markers (100). Thickening agents such as acacia, gelatin, xanthan gum, cellulose derivatives, polyvinyl pyrolidone are added into the external aqueous phase to increase viscosity and stability of the system (88,105). It is also possible to enhance stability of the systems by adding aluminium salts and fatty alcohols into the oil phase (106).

2.3.2.5.2. Physical Factors

2.3.2.5.2.1. Shear

Since multiple emulsions are considered to be thermodynamically unstable, shear rate is an important factor in the manufacture of multiple emulsions. Usually, a high agitation speed during primary emulsification and low agitation speed during secondary emulsificatoin process is required. If the emulsion droplets undergo high shear for a long period of time, the droplets

formed break into smaller droplets and thus stability is affected. On the contrary, if the speed is too low, droplets formed are large leading to creaming and/or coalescence. Therefore, an optimal speed of agitation is required during each step. It has been noted that some of the internal phase is unavoidably lost into the external phase whatever technique is used (107).

2.3.2.5.2.2. Volume Fraction

A stable w/o/w multiple emulsion can be prepared by using the internal volume fraction ($\phi_{w/o/w}$) in a range of 20–90 % (107) . However, optimal range is 50-80 % (107). Maximum stability of multiple emulsion is achieved when the droplets are closely packed. When the volume fraction is high, the oil droplets are so closely packed that movement and swelling of the droplets are difficult, but sometimes the spherical globules become deformed.

2.3.2.5.2.3. Ratio of Emulsifiers

Usually, a primary emulsifier is required in greater quantity than the secondary emulsifier to form a stable multiple emulsion. Generally, the secondary emulsifier is 1/5 of the primary emulsifier but this quantity may be decreased to a ratio of 1:1 (79). If the concentration of the hydrophilic emulsifier is higher in a w/o/w multiple emulsion, the primary emulsifier may migrate into the secondary emulsifier micelles thus decreasing the stability of the system (107).

2.3.2.5.2.4. Temperature

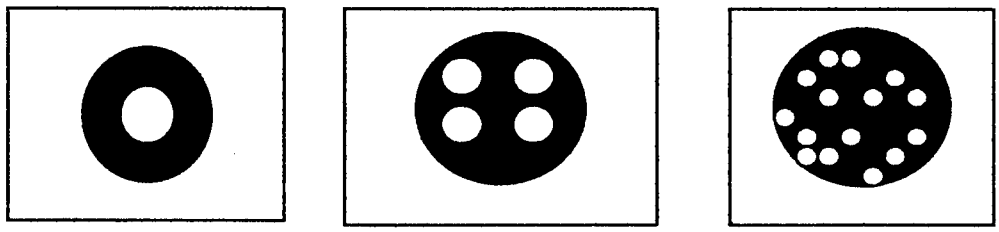
There should be a strict control on the temperature during manufacturing. Manufacturing temperatures are approximately 70^0C for primary emulsification and 25^0C for secondary emulsification (79). Temperature has an influence on the interfacial tension, the lipophilicity of the emulsifier and the miscibility of the two phases (107).

2.3.3. Characterization

2.3.3.1. Types of Droplets

In practice, it is possible to obtain three different types of droplets in multiple emulsions (**Figure 2.4.**) (97). All of these types may or may not exist in one multiple emulsion. The simplest system, type A, consists of relatively small multiple droplets with an average diameter of 8.5 μm containing a few, usually only one, relatively large internal aqueous droplets. Most of the multiple globules

contain only one internal aqueous droplet. In type B emulsions, multiple droplets are of larger sizes usually with a mean diameter of 19 μm containing smaller but numerous internal droplets. The diameters of the internal aqueous droplets may be in the range of 5-30 μm. In type C emulsions, the multiple droplet size is larger, i.e. 25 μm, with a vast number of internal aqueous droplets. The internal aqueous globules are uncountable due to the drawback in the resolution of the internal aqueous droplets. This system retards the release of entrapped materials to a much greater extent than emulsions A and B (97).



Type A multiple Type B multiple emulsion Type C multiple emulsion

Figure 2.4. Different Types of Multiple Droplets

2.3.3.2. Yield Value

Yield of preparation is a measure of the ratio between the amount of a substance entrapped in the internal droplets and external phase, or the percentage of multiple droplets obtained immediately after preparation.

All the evaluation methods are based on quantitative measurements of the amount of a marker released from the internal phase to the external phase. Such markers are usually electrolytes (NaCl, $MgSO_4$, ephedrin HCl) (102,104,135); water soluble additives such as glucose; fluorescent probes which may be determined by potentiometric titrations (135); specific electrodes (136); microanalysis (121); electrical conductivity (102,1136,137) or fluorescent measurements (91).

The yield of preparation can be expressed as (138)

$$M_{in} * 100 / M_{in} + M_{out}$$

where M_{out} and M_{in} are the amounts of marker in the external and internal phases, respectively. Therefore, the yield of preparation is the percentage of a marker released at a short time after preparation.

Determination of the released marker can be followed directly using the multiple system (102), after dialysis against water (121), or after filtration of the multiple droplets (139). With the dialysis tubes, the measurement indicates not only the preparation yield but also the amount released during the dialysis period which can be as long as 20 hours (140). Markers may affect the formation of multiple emulsions with an increase in osmotic pressure, interactions with the surfactants, etc. Therefore it is advisable to conduct microscopic observations as a qualitative guide for the preparation yield. Some investigators have used the microscopic observation as a quantitative measure of the preparation yield by counting the empty and loaded emulsion droplets (110). However, since this method is time consuming, it is not recommended for routine use in practical applications of multiple emulsions (141,142). Matsumoto and coworkers (133) used a relation between the viscosity and the dispersed phase volume fraction to estimate the stability of multiple emulsions. When measurements were conducted immediately after preparation, yield of preparation was obtained.

A direct method was recently suggested by Eads et al (143) using unique direct measurements by nuclear magnetic resonance (NMR).

The 1st stage in the design of multiple emulsions has to focus on developing a suitable method to measure the yield of preparation. This method can also be used to determine the stability and release characteristics of a specific multiple emulsion system.

2.3.3.3. Droplet Size Analyses

Multiple droplets have an average diameter of 5-20 μm (108). Globule size analysis can give no direct information about the multiple structure while visualization gives direct information about the multiple structure. Multiple emulsions which are liquid/liquid systems have two different globule size populations. It is possible to characterize both populations by different techniques. These techniques are:

- Counting of particles (109),
- Optical microscopy (110),
- Electron microscopy (111),
- Light scattering and diffraction methods (112).

2.3.3.3.1. Counting of Particles

The Coulter counter system is used for this method which deals with the passage of particles through an orifice. The coulter counter system works on Ohm's Law to measure the resistances generated in electrodes when a particle passes through an orifice (109).

The limitations of this method are the requirements of conducting media and the hyperosmotic conditions that may affect the results (109).

2.3.3.3.2. Optical Microscopy

With the help of an optical microscope, particles around 0.5 μm can be measured. With the help of image analysis techniques, qualitative as well as quantitative characterization of the particles are possible (109).

2.3.3.3.3. Electron Microscopy

With the help of an electron microscope, it is possible to visualize emulsions in a concentrated form whereas with the optical microscope, the emulsions need to be diluted before their analysis. It is not possible to observe a sample of emulsion directly under an electron microscope. The sample to be analyzed is freeze fractured (111).

2.3.3.3.4. Light Scattering Methods

In those methods, when the light beam collides with a particle, it is scattered (**Figure 2.5.**). The scattering of light includes refraction, reflection and diffraction. The most frequently used wavelengths, i.e. wavelengths between 400 and 800 nm, lie within the visible spectrum. The main advantage of this method is the possibility of selection of medium in which the measurements can be performed.

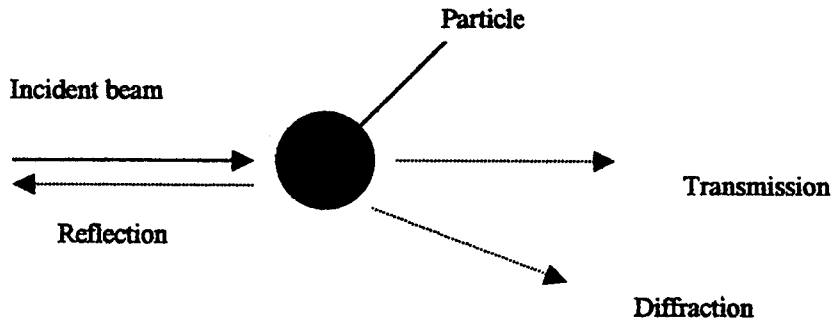


Figure 2.5. Scattering of Light

2.3.4. Stability

Even though the mechanisms of instability in multiple emulsion systems are complex, theoretical predictions appear to agree with the experimental results (94). Multiple emulsions of w/o/w type may deteriorate by several possible mechanisms (94);

1) Shrinkage or swelling of the internal drops due to the osmotic pressure gradient on both sides of the oil film which acts as a semipermeable membrane,
2) Coalescence of the internal aqueous droplets within the oil phase,
3) Coalescence of the oil globules whether simple or multiple,
4) Rupture of the oil layer on the surface of the internal droplet.

Several potential applications of multiple emulsions have led many researchers to overcome the inherent instability of the multiple system. The presence of electrolytes is one of the most important factors playing a role in the stability of the system (107). The effects of electrolytes are two fold:

- First, to promote an osmolar balance between the internal and external aqueous phases,
- Second, to increase the stability of the system due to the phenomenon of salting out. There is a competition between electrolyte and emulsifier for water molecules. Lipophilic emulsifier becomes less hydrophilic and push off at the interface. This would result in a rigid interfacial layer and form a more effective mechanical barrier.

One of the approaches to the stabilization of multiple emulsions is by polymerization of monomers dissolved in the aqueous phases (113). Surfactants from a series of modified polymerizable nonionic surfactants can form a crosslinked membrane after adsorption at the interface. Such an interfacial surfactant network can restrict the migration of surfactants (114). A stabilizing film can be formed through the interfacial interaction between macromolecules such as albumin or a polyanion such as polyacrylic acid in the internal aqueous phase and nonionic lipophilic surfactant in the oil phase (115). The preparation of multiple w/o/w emulsion system with improved stability due to the formation of interfacial complex films between acacia, gelatin, polyvinylpyrolidone and sorbitane monooleate has been reported (93). Possible stabilization could occur by using bovine serum albumin in the internal phase and sorbitane monooleate in the oil phase (112, 116).

The osmotic pressure gradient between the two aqueous phases may lead to a dynamic change in the volume of the internal aqueous phase due to the migration of water through the oily layer (117). At higher concentrations of electrolytes, extreme osmotic imbalance exists and the internal water is lost rapidly by the rupture of the oil layer. At lower concentrations of electrolytes, if the osmotic pressure is higher in the internal aqueous phase, water may pass to this phase resulting in the swelling of the internal droplets which eventully burst releasing the contents. Reversely, if the osmotic pressure is higher in the external aqueous phase, the result will be the shrinkage of the internal droplets. Besides electrolytes, proteins, glucose or glycerol may be used to control the rate and amount of materials transferred from one phase to the other (107).

In w/o/w emulsion systems, true osmotic flow occurs, i.e. the oil layer is impermeable to solute but permeable to solvent molecules (118). Some researchers have calculated the water permeability coefficient using different methods (117, 119). According to the studies on lipid membrane system the value for the permeation coefficient P_0 was calculated as

$$\phi_w = P_0 A (g_2 c_2 - g_1 c_1)$$

where, ϕ_w is the mole flux of water across the oil layer; A is the surface area of oil layer; g and c are the osmotic coefficient and the concentration of glucose,

respectively (117). Subscriptions of 1 and 2 refer to the medium side and the compartment side of the oil layer.

Transfer of the drug to the external aqueous phase can occur either by total breakdown of the multiple droplets or by diffusion of the unionized drug through the oil layer (94,120). It has been suggested that the yield of the w/o/w emulsion system can be measured by dialyzing the solute in the internal aqueous phase against distilled water (121). However, if the solute can permeate the oil layer, there is no way to distinguish between the solute diffused and the solute leaked out as a result of vesicle destruction. Using the amount of tracer liberated, the yield of multiple emulsion can be calculated using

$$R = Y_0 - Y_1 / Y_0$$

Where, R is the yield value, Y_0 is the initial amount of tracer in the internal phase and Y_1 is the amount in the external phase. If R=1, the stability of the emulsion is optimal.

2.3.4.1. Mechanisms of Drug Release

There are several possible mechanisms by which materials may be transferred across the oil layer in a w/o/w system. The most possible mechanism of drug liberation is by diffusion. This depends on the nature and concentration of the active compound. Florence and Whitehill (94) have suggested that release of unionized materials from w/o/w multiple emulsion systems follow 1st order kinetics, Fick's law being obeyed:

$$dCe/dt = DA/dx \ (Ce-Ci)$$

where, Ci= concentration of drug in the internal aqueous phase, Ce= concentration of drug in the external aqueous phase, D= diffusion coefficient of drug in the oil layer, dx= diffusion layer thickness and t= time. When a trapping agent is used in the internal aqueous phase, the material becomes ionized and Ci becomes negligible. Therefore, the equation above can be written as:

$$dCe/dt = - DA\ Ce/dx = -K\ Ce$$

and by integration

$$Ce = Ce\ e^{-kt}$$

The rate constant, K, is a function of the diffusion coefficient of the drug, the area between the external phase and the liquid membrane, and the membrane thickness.

Brodin and Frank (122) have interpreted the release of drug from the o/w/o multiple emulsion system. Release pattern was complex particularly in systems containing higher concentrations of the drug. An initial rapid release (α-phase) followed by a much slower release rate (β-phase) was obtained. The rapid onset of α–phase corresponded to the release from an oil suspension which reflected the presence of drug particles in the external oil phase due to the loss from the internal oil phase during manufacture of the emulsions. The much slower release during β–phase is the release from the internal oil phase. Yang and Rhodes (123) also found a two phase mechanism for the transport of the solute from the external aqueous phase to the internal aqueous phase.

Although diffusion seems to be the most important mechanism of transport in multiple emulsion systems, there is an evidence that ionized materials may pass across the oil layer in the w/o/w emulsion. Kita et al (124) suggested two possible mechanisms for the permeation of water through the oil layer: 1st of these is the inclusion of water in mixed, inverse micelles of hydrophilic and hydrophobic surfactants; the other mechanism is when water molecules may diffuse across very thin lamellae of surfactants formed where the oil layer is very thin. The 3rd possible transport mechanism is carrier mediated transport (125). This involves either incorporation of some material into the internal aqueous phase or membrane which reacts with the permeating compound to render it liposoluble. The carrier compounds effectively 'pump' the permeating compound across the membrane. Kopp et al (125) quoted the example of stearic acid (HSt) used as a carrier for Cu^{++} ion according to mechanism

$$Cu^{++} + 2HSt \leftrightarrow 2H^{+} + CuSt_2$$

The copper ion reacts with two stearic acid molecules in the oil membrane, releasing two protons, and is transferred as copper stearate across the oil phase and exchanged against two protons at the inner interface. The protons are then transported back as stearic acid across the oil layer and exchanged against the

copper ion at the outer interface. Using such a system, copper ion is transported against a concentration gradient.

A 4th and possibly much less important transport mechanism is the solubilization of minute amounts of the internal phase in the membrane phase resulting in the passage of very small quantities of materials (94).

2.3.4.2. Instability Mechanisms

Florence and Whitehill (94) proposed some of the possible instability mechanisms considering the w/o/w system as shown in the **Figure 2.6.**

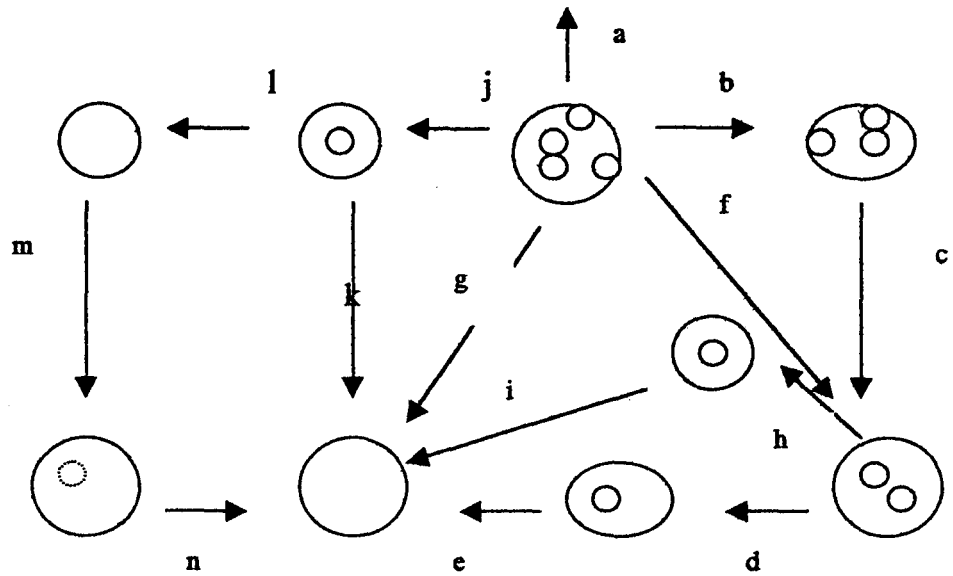


Figure 2.6. Some Possible Pathways for the Breakdown of w/o/w Multiple Emulsion

The multiple (oil) droplets may coalesce with the other oil drops, simple or multiple (a); the internal aqueous droplets may be expelled individually (b, c, d, e) or more than one may be expelled (f); they may be less frequently expelled in one step (g); the internal globules may coalesce before being expelled (h, i) , (j, k); water may pass by diffusion through the oil phase gradually resulting in the shrinkage of the internal droplet (l, m, n). Whether all these mechanisms occur in all systems is not clear, neither is the relative importance of each mechanism in different w/o/w systems. On the other hand, this may be simplified and a combination of the above events may take place.

2.3.4.3. Methods Used For the Estimation of the Stability of Multiple Emulsions

Several techniques are used to detect and quantify breakdown of multiple droplets in multiple emulsions.

2.3.4.3.1. Microscopy

Measurement of the size and distribution of multiple droplets gives a good idea for the stability. This can be achieved by using light microscopy (110, 112, 119) or electron microscopy (126, 127) for a definite period of time.

2.3.4.3.2. Centrifugation

Samples of freshly prepared emulsions are subjected to the centrifugal stress of 1000 rpm for 60 minutes. The cream volume or the separation of phases at a given time is taken as a measure of the physical stability of emulsion (128).

2.3.4.3.3. Thermal Analysis

Thermal stability tests are carried out at below 0^0C (129), 4^0C$\pm1^0$C, 25^0C$\pm1^0$C and 40^0C$\pm1^0$C to investigate any phase seperation and especially creaming (130).

2.3.4.3.4. Conductivity

Conductivity differences arise when an emulsion creams and the proportion of oil increases in the upper part of emulsion and the proportion of water increases in the lower part of emulsion. By measuring the conductivity differences in the upper and lower parts as a function of storage time, instabilities can be determined (131). Conductometric analysis can also be achieved by dilution of the multiple emulsion containing an electrolyte with an isotonic solution and measuring the conductivity over a period of time (110,127).

2.3.4.3.5. Viscometric Methods

Since multiple emulsions are known to be viscoelastic materials exhibiting the characteristics of both the liquid and solids, their rheological properties must be determined (132). Due to their great potential for characterization and description, rheological analyses are absolutely necessary to define and optimize stable multiple emulsions. These analyses allow the following of changes in multiple emulsions induced by aging, shear and temperature. Moreover, the

rheological properties allow us to describe and control the disruption mechanisms of the oily globules, which occur either by an osmotic swelling or simple shear.

The viscosity of the w/o/w emulsions can be expressed by Mooney's equation as (133):

$$\text{In } \eta_{rel} = a(\phi_0-\phi_w)/ [1-\lambda(\phi_0+\phi_w)]$$

where, η_{rel} is the relative viscosity of the sample; ϕ_0 and ϕ_w are the volume fractions of the oil and aqueous phases, respectively; a is the shape factor; and λ is the crowding factor (134). These constants obtained experimentally show an irregular fluctuation in the range of 2.3 and 1.9 for a, and 0.5 and 1.9 for λ. The volume fraction of the internal aqueous phase ϕ_w can be obtained using the equation above

$$\phi_w= [(1-\lambda\phi_0) \text{ In } \eta_{rel}-a\phi_0] / (a+\lambda \text{ In } \eta_{rel})$$

Volume fraction of the oil phase, ϕ_0 , remains constant after rupture of the oil layer while volume fraction of the inner aqueous phase, ϕ_w , decreases with the increase in number of ruptures. Therefore, the decrease in the volume fraction, ϕ_w reflects the viscosity of the w/o/w emulsion. Examination of the viscosity changes at a definite period of time may determine the stability of the multiple system (113, 133, 134).

2.3.5. Cosmetic Applications

Cosmetics mean any article intended to be used by means of rubbing, sprinkling or by similar application to the human body for cleaning, beautifying, promoting attractiveness altering the appearance of the human body, and for maintaining health of the skin and hair, provided that the action of the article on the human body is mild (144).

Cosmetic products must comply with the requirements in order to respect the integrity of *Stratum corneum* which is a very impermeable barrier (145, 146).

Aesthetic considerations, such as pleasant odour and colour, good spreadability and smooth skin feeling are also very important for the cosmetic products. A long shelf life, usually two years, is also required. This means no phase separation, discolouration, mal odour development or bacterial growth during this period of time (147).

Due to the structure of multiple emulsions, they can offer promising applications in pharmaceutical, food and cosmetic industries. Multiple emulsions can ensure:

- complete protection of the entrapped drug,
- different drug release characteristics: immediate and prolonged.

2.3.5.1. Benefits of Multiple Emulsions in Cosmetic Formulations

Common properties of both w/o/w and o/w/o multiple emulsions are of great interest to the cosmetic industry. The reasons include:

- high capacity of entrapment of materials (79),
- possible protection of fragile substances encapsulated in the internal compartment (3),
- possibility to dissolve incompatible substances in the internal and external separate compartments (2),
- prolonged release of active substances incorporated into the multiple droplets (127).

In addition to these well known properties, others seem more related to special textures presented by these systems:

- a pleasant skin feel like that of o/w emulsions combined with the well known moisturizing properties of w/o emulsions for w/o/w systems (140),
- the continuous phase can be gelled to provide the right consistency (148).
- no need to incorporate into a delivery system (94).

2.3.5.1.1. Original Textures

Among the current trends in the cosmetic industry, texture research is a very important tool to improve appeal to the consumer. Multiple emulsions, with their particular rheology, are good candidates for providing an interesting feeling: efficient, light, non greasy textures. For example, o/w/o emulsions can be of great interest in the field of sun protection products: continuous oil phase emulsions are well known to resist washing off and have good spreadability on the skin for lipophilic UV filters (2). However, for simple w/o emulsions, the addition of waxes, which are necessary to increase emulsion stability and viscosity, have the

incorporation of ingredients which have incompatibilities with silicone oils have been studied (150). The incorporation of micronized TiO_2 in the o/w/s multiple emulsion for sun protection purpose was investigated by Semenzato et al (2). It was found that the efficacy of UV absorption of these ultrafine particles depended mainly on their degree of solvation in a suitable medium. The best SPF, i.e. 35 was obtained with organically treated hydrophobic TiO_2 dispersed in organic oils such as esters or hydrocarbons. Silicon oils are also very useful in providing good spreadability and water resistance. Nevertheless, these oils are not very good solvents for TiO_2 and agglomeration of particles may cause some problems (150). Thus, a good solution is to keep TiO_2 dispersion by encapsulating it in the internal oil phase of a o/w/s multiple emulsion.

2.3.6. Fields of Applications

Multiple emulsion systems have been designed to be applied cosmetically because of their ease in skin application and encapsulation character. Skin care can be maintained over twenty hours; sustained release and protection of fragrances and other sensitive materials can be achieved. Multiple emulsions have been claimed to be useful when applied as sunscreen, moisturizing, nutritive and protective hand creams, make-up cleansers, shaving creams, antiperspirants and perfume preparations by incorporating the active agents either in the internal or external phase (151-161).

2.4. MACADAMIA NUT

Macadamia nuts contain oil more than 75 % of their weight, 9.0 % proteins, 9.3 % carbohydrates, 1.5 % moisture, 1.6 % mineral matters and 2.0 % fiber (4, 5). The kernels of macadamia contain vitamin A1, B1, B2, niacin and essential elements such as calcium, iron, phosphorus, magnesium and potassium. The oil is a triglyceride oil and contains primarily monounsaturated fats up to 80-84 %. Macadamia oil contains the highest percentage of monounsaturates when compared to both olive and canola oils (5).

Table 2.4. Contents of Unsaturated and Saturated Fats of Different Oils

OIL TYPE	PERCENTAGE OF UNSATURATED FATS		PERCENTAGE OF SATURATED FATS
	Poly-	Mono-	
Macadamia oil	4	84	12
Almond oil	25	65	10
Animal frying oil	5	45	50
Butter	7	36	57
Canola oil	30	63	7
Olive oil	10	76	14
Palm oil	10	39	51
Peanut oil	36	45	19
Pecan oil	34	55	11
Safflower oil	77	14	9
Soyabean oil	62	23	15
Sunflower oil	66	23	11

The macadamia nut based diet, high in monounsaturated fat and the moderately low-fat diet both have potentially beneficial effects on cholesterol and LDL in serum and reduce the risk of a heart attack (162, 163). In the past, many have avoided nuts because of their high fat content. The dietary approach to stop hypertension (DASH), however, recommends regular consumption of this food along with seeds and dried beans (4-5 servings per week). Nuts are nutrient dense and most of their fat is unsaturated. They are also the best natural source of Vitamin E and are relatively concentrated repositories of dietary fiber, magnesium, potassium, and argnine, the dietary precursor of nitric oxide (163). Human feeding studies have demonstrated a reduction of 8-12% in low density lipoprotein (LDL) when almond and wall nuts are substituted with more traditional fats. Other studies show that macadamias and hazelnuts appear at least as beneficial as fats in commonly recommended diets. The idea of weight gain due to daily consumption of modest quantities of nuts is not certain, since preliminary data suggest that this is unlikely (163). Four of the best and the largest cohort studies in nutritional epidemiology have reported that eating nuts

frequently is associated with a decreased risk of coronary heart disease with the order of 30-50%. The findings are very consistent in subgroup analyses and unlikely to be due to confounding. Possible mechanisms include reduction in LDL cholesterol, the antioxidant action of vitamin E and the effects on the endothelium and platelet function of higher levels of nitric oxide.

2.4.1. Macadamia Plant

Macadamia oil is obtained from the nuts of macadamia trees (*Proteaceae family*) (164). Common names of the trees are, Australian nut and Queensland nut. Species are "smooth shelled macadamia" called *Macadamia integrifolia* and "rough shelled macadamia" called *Macadamia tetraphylla* (164). *Macadamia ternifolia* is also the name used for *M. integrifolia*. *Macadamia integrifolia* is native to Australian and Hawaii islands. It grows in rain forests and close to streams. *Macadamia tetraphylla* is native to Southeastern Queensland and Northeastern New South Wales. Macadamias are large spreading evergreen trees reaching 30-40 feet height (164). Macadamia has proteoid roots, dense cluster of short lateral rootlets in well defined rows around the parent root axis. The two species are fairly easily distinguished by their foliage. The leaves of *M. integrifolia* are 8-11 inches in length and occur usually in whorls of 3. The spiny, often sessile leaves of *M. tetraphylla* usually appear in whorls of 4 and may grow to 20 inches long. *M. integrifolia* has creamy white flowers borne in clusters 6-12 inches long, while the flowers of *M. tetraphylla* are cream-coloured or pink and borne in clusters up to 15 inches long (164). Macadamia nuts have a very hard seed coat enclosed in a green husk that splits open as the nut matures. This seed coat of *M. integrifolia* is smooth . It holds a creamy white kernel containing up to 80 % oil and 4 % sugar. When roasted, it develops a uniform color and texture. Although *M. tetraphylla* is a rough shelled macadamia, the seed coat of some cultivars are smooth, while others are rough and pebbled. The quality of the kernels of *M. tetraphylla* are also variable. The oil content ranges from 65 % to 75 % and sugar content ranges from 6 % to 8 %. These factors result in variable colors and texture when the nuts are roasted under the same conditions (164).

2.4.2. Macadamia Nut Oil

MIAMP1 is a low molecular weight cysteine-rich antimicrobial peptide isolated from the nut kernels of *Macadamia integrifolia* (165, 166). On the other hand, no antimicrobial activity of macadamia oil in a concentration of 2 % (v/v) has been reported in another study (167). Macadamia nut ingestion can cause immediate-type 1 allergic reactions. Hypersensitivity reactions with macadamia nuts have also been reported (168).

Phenolic compounds in macadamia nuts and shells were identified and their antioxidant activities were evaluated in a refined macadamia nut oil (169). Thin layer chromatography of oil extracted from macadamia nut kernels and shells indicated the possible presence of catechol, pyrogallol, and 3,4,5-trihydroxy phenolic compounds (169). In a similar report by Rosenthal et al (170), accelerated oxidation tests indicated that the kernels of *M. integrifolia* were more resistant to rancidity than those of *M. tetraphyla*. The oxidative stability index of macadamia nut oil is high compared to other naturally derived oils (171).

The chemical composition of the fatty acid portion of macadamia oil has been reported by Alban Muller International (172) as:

Oleic acid	54-68 %
Palmitoleic acid	16-23 %
Palmitic acid	7-10 %
Stearic acid	2-5.5 %
Arachidic acid	1.5-3 %
Linoleic acid	1-3 %
Eicosenoic acid	1-3 %

Approximately the same ingredients has been reported by Vega et al (173). Palmitoleic acid at such a high concentration is rarely found in vegetable oils. This fatty acid is mostly found in fish oils. Palmitoleic acid is found in macadamia oil in concentrations as high as 21% (174). This fatty acid is secreted by young children's sebaceous glands giving them pulp and dewy skin. It almost

disappears from the sebum by aging (175). It helps to recapture the skin of childhood. It effectively hydrates dry and rough skin and reduces the appearance of the fine lines including those around the eyes. It is ideal for use where penetration and lubrication are essential and provides amazing slip for massaging (175). It shows excellent absorbancy with protective barrier which does not clog the pores of the skin. It is non toxic, non allergenic and non-staining. It is easily removed by soapy water.

It is well known that lipid peroxidation damages cell membrane. It was determined that the presence of palmitoleic acid played a role in this protection (176). Use of macadamia nut oil may protect cells in a similar manner. This may be particularly true in skin exposed to excessive sunlight. It is also an excellent emollient and possesses a good afterfeel and superior spreading coefficient (176).

Due to its excellent properties, it is being used in a variety of cosmetic products including lipsticks, skin conditioners, hair, antiacne, sunscreen and bath preparations, nail lacquer removers, shampoos, shower oils, and other skin preparations. Macadamia nut oil can be used in any cosmetic product as an active principal or as a carrier in the oily phase without any limit (177-185).

2.5. ASCORBIC ACID (VITAMIN C)

Vitamin C or ascorbic acid is a white or almost white, crystalline powder or colorless crystals; discolor on exposure to air and moisture; freely soluble in water, soluble in alcohol, and practically insoluble in ether (186). Its chemical structure is given in **Figure 2.7.**

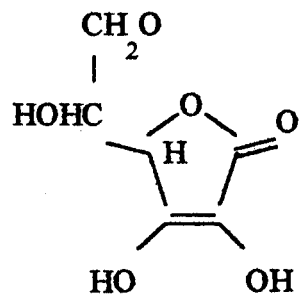


Figure 2.7. Chemical Structure of Ascorbic Acid

It is found at quite high concentrations in the aqueous fractions of many animal tissues including the spinal cord, lungs and eyes. In plants, some fruits may contain more than 1 %. It normally occurs at a level of about 0.1 mM in human blood plasma. Because of its enediol structure, it displays a rather low first pKa (about 4.2) and accordingly exists almost entirely as a monoanion in most tissues.

2.5.1. Physico-Chemical Characteristics

Ascorbic acid has important physiological effects on skin, including inhibition of melanogenesis, promotion of collagen biosynthesis and prevention of free radical formation (187-191), all closely related to the well-known antioxidant properties of this compound (192-194). Vitamin C therefore plays an important role in skin aging and is considered as an interesting ingredient of cosmetic skin care products (192).

The formulation of products with ascorbic acid is of challenge because this substance is extremely unstable (195-197) : it undergoes oxidation, especially in aerobic conditions (copper or heavy metals catalize this reaction) (196, 197) and with light exposure (198, 199). These reactions occur rapidly at standard conditions and the compound degrades itself irreversibly to a biologically inactive form (2, 3-diketo-L-gluconic acid). To overcome this problem, vitamin C is chemically modified by esterification of the hydroxyl group using long chain organic or inorganic acids (195, 200). Two derivatives are widely used in topical formulations: ascorbyl palmitate, a fatty acid ester with lipophilic properties (200); and magnesium ascorbyl phosphate (VC-PMG), an inorganic water-soluble acid ester (201).

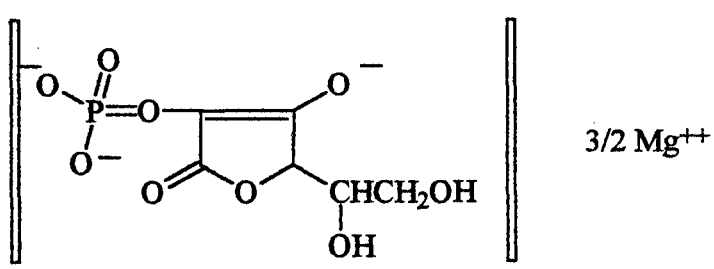


Figure 2.8 Chemical Structure of Magnesium Ascorbyl Phosphate

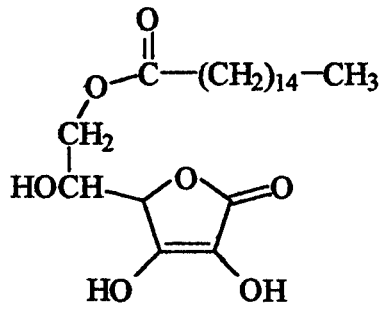


Figure 2.9. Chemical Structure of Ascorbyl Palmitate

2.5.2. Stability

The stabilities of ascorbic acid, ascorbyl palmitate and magnessium ascorbyl phosphate (VC-PMG) in both standard solutions and topical formulations were investigated by direct RP-HPLC analysis after sample dilution with a suitable aqueous-organic solvent mixture (202). The results have shown that the two vitamin C derivatives were more stable than ascorbic acid itself. The ascorbyl esters have shown significant differences. Esterification with palmitic acid in 6-position did not prevent hydrolysis of the molecule, either in solution or in emulsion. Only the special preparation of products with high viscoelastic properties was able to reduce the typical behaviour of this compound (202). Conversely, the introduction of phosphoric group in 2- position protected the molecule from break up of the enediol system, confirming VC-PMG as a very stable derivative of vitamin C that may easily be used in various types of cosmetic products (202).

Several O/W microemulsions, O/W and W/O emulsions and a W/O/W multiple emulsion were prepared using non-ionic, non-ethoxylated skin compatible emulsifiers (203). Vitamin C has been added to the emulsified systems and its stability against oxidation was studied at 45°C in aerobic conditions and compared to that in aqueous solutions at different pH values (203). All emulsified systems provided protection for vitamin C since its degradation rate was slower in emulsified systems than in aqueous solutions which increased with increasing pH. The highest protection of ascorbic acid was when it was dissolved in the internal aqueous phase of the W/O/W multiple emulsion (203).

2.5.3. Effects on Skin

2.5.3.1. Antioxidant Activity

Ascorbic acid is a potent reducing agent whose low redox potential of about 280 mV means that it has the thermodynamic potential to react with almost all other oxidizing free radicals (204). Experimentally, it reacts rapidly with even weak oxidizing agents, including the free radicals $\cdot O^-_2$ and $\cdot OOH$, as shown by pulse radiolysis and flash photolysis studies (205). The rate constant for reaction of superoxide with ascorbate at nearly neutral pH has been reported to be in the range of $2.7x10^5$ - $6.5x10^5$ L/mol sec , which is greater than that of other biomolecules except glutathione, metalloproteins, a few polyphenols and quinones (206,207). Ascorbate radical, ($\cdot A^-$), can terminate a chain reaction by disproportionating into the non-radical product ascorbate dehydroascorbate. However, the reaction efficiency is partly ameliorated by the ability of ascorbate to produce superoxide upon its own oxidation by molecular oxygen.

$$AH + \cdot O_2^- \rightarrow \cdot A^- + O^2 + H^+$$

$$AH + O_2 \rightarrow \cdot A + \cdot O^-_2 + H^+$$

Ascorbic acid reacts with typical peroxide radicals in water with a rate constant of $2x10^6$ L/mol sec. (208). This value is approximately 4 times lower than the rate constant of vitamin E. The number of peroxide radicals trapped per mole of ascorbate is in the range from nearly 2 at low ascorbate concentrations to almost 0 at high concentrations (209). This is due to self termination reactions that compete with radical trapping at higher ascorbate levels.

Ascorbic acid quenches singlet oxygen with a rate constant of about 10^7 L/mol sec (210). The products include oxalic acid $(HOOC)_2$ and threonic acid $HOOC\text{-}(CHOH)_2\text{-}CH_2OH$ (211). It is probable that the quenching ability of ascorbate is not particularly important in the aqueous environment where it is found, since $^1O_2$ has such a short life time in water in any case.

2.5.3.2. Photoprotection

Vitamin C (l-ascorbic acid) functions as a biological co-factor and antioxidant (6-8). It has been touted as important in treating pathologies ranging from cancer to the common cold, presumably due to its antioxidant functions (212). Ultraviolet-radiation damage to the skin is partially due to the generation of oxygen free radicals. Topical application of vitamin C was shown to significantly elevate the cutaneous level of this vitamin in pigs (213). This protects the skin from UVB damage, as measured by erythema and sunburn cell formation. This biochemical protection is due to the reducing properties of the molecule (213). Other investigators have shown that topical vitamin C treatment can significantly retard UVA-mediated damage to the skin, and may thus serve as a biological-origin, broadspectrum photoprotectant and/or anti-inflammatory agent. The investigators have also provided evidence on the possible depletion of skin's vitamin C level after UV irradiations (214). This would lower the skin's protection mechanism leaving it at risk to poorer healing after photoinduced damage. The prophylactic activity of topical vitamin C therapy may prove to be valuable in decreasing UV-induced trauma as well as other skin pathologies. A subsequent study on humans showed that topical vitamin C treatment has reduced UVB radiation-induced erythema (214). This result suggests that free radicals are involved in UV damage to human skin and that vitamin C can provide prophylactic photoprotection.

Topical vitamin C is claimed to inhibit ultraviolet-radiation-induced damage to porcine skin (6). Photoprotective effects of topically applied antioxidants, when applied before UVR exposure are well known. However, their protective effects when applied after UV exposure is less established. In a randomized, double blind, placebo-controlled human study, the short-term photoprotective effects of different antioxidants and of their combinations were evaluated when applied after UV exposure (215). No significant protective effect of meltonin, vitamin C or vitamin E were obtained. UV-induced skin damage is a rapid event, and antioxidants possibly prevent such damage only when present in relevant concentration at the site of action at the beginning and during oxidative stress (215).

Natural antioxidants, are now-a-days being used in photoprotection. Two of the best known antioxidants are vitamin C and vitamin E, both of which have been shown to be effective in different models of photodamage (216). Very little has been reported, however, on the combination of the two nor have there been detailed studies on the ability of these antioxidants to augment commercial sunscreen, protection against UV damage. Darr et al (216) reported that vitamin C is capable of additive protection against acute UVB damage when combined with a UVB sunscreen. Vitamin C is significantly better than vitamin E at protection against a UVA mediated phototoxic insult in the animals while the combination is only slightly more effective than vitamin C alone (216).

The effect of stable vitamin C, magnesium-L-ascorbyl-2-phosphate (MAP), administered after acute and chronic exposure to UVB irradiation were investigated using hairless mice and the results suggested that postadministration of MAP delays progression of skin damage (217). It is presumed that MAP, once converted to ascorbic acid exhibits such inhibitory effects by scavenging hydroxyl and lipid radicals generated as a direct or indirect result of UVB exposure (217).

Acute adverse effects of ultraviolet radiation in human include sunburn, photosensitivity reactions and immunological suppersion (218). Chronic exposure to UV light, particularly the UVB (290-320 nm) component of UV radiation, and certain environmental chemicals increase the risk of nonmelanoma skin cancer and play a major role in cutaneous aging. The lipid peroxidation (LPO) of biomembranes, mediated by reactive oxygen species and free radicals, is one of the major causes of cellular damage induced by UV radiation and toxins. Antioxidants such as vitamin E, vitamin C and melanins are reactive oxygen and radical scavengers, thereby minimizing the light and toxin induced tissue destruction. The influences of 8 biotechnologically produced polyphenolic melanins on the LPO of microsomal membranes in comparison to alpha-tocopherol, ascorbate and synthetic melanin were tested (218). All biomembranes showed better inhibition of peroxidative damage than synthetic melanin. Three of the tested drugs inhibited the LPO at least as effectively as vitamin C and vitamin E. The combination of the most effective biomelanin with both vitamin C and

vitamin E resulted in higher LPO inhibition than caused by each agent alone. Data obtained shows that biomelanin is a potent inhibitor of the peroxidative destruction of biomembranes, indicating that these compounds may be useful antioxidative agents in cosmetic preparations (218).

2.5.3.3. Others

The analysis of epidermal lipids reveals that (glucosyl) ceramide profiles in various human skin are different from those of native tissues (219). The main difference is the reduced content in skin equivalents of ceramides 4-7 and especially the very low content of the most polar ceramides 6 and 7 which contain hydroxylated sphingoid base and/or fatty acid. To fascilitate hydroxylation, the culture medium was supplemented by vitamin C and vitamin E. Although, lipogenesis was not affected in vitamin E supplemented medium, at the vitamin C supplemented medium, the contents of glucosylceramides and of ceramides 6 and 7 were markedly increased. The improvement of the lipid profile was accompanied by an apparent improvement in the barrier formation. These results indicate the key role of vitamin C in the formation of *Stratum corneum* barrier lipids (219).

The *Stratum corneum* has been recognized as the the main cutaneous oxidation target of atmospheric ozone (O_3), a major part of photochemical smog. Weber et al (220) reported the presence and distribution of vitamin C, glutathione and uric acid in murine *Stratum corneum* and evaluated the susceptibility of these agents to acute environmental exposure to O_3. Ozone was applied at different concentrations on hairless mice. The concentrations of hydrophilic antioxidants were measured before and after the exposure. It was found that vitamin C was decreased to 80 % of its pretreatment contents, glutathione to 41 % and uric acid to 44 %. This report demonstrates the previously unrecognized role of hydrophilic antioxidants in the *Stratum corneum* and provides further evidence that O_3 induces oxidative stress on this outer skin layer.

Postoperative erythema is a universal and problematic side effect of cutaneous carbon dioxide (CO_2) laser resurfacing (221). A study was conducted in order to determine the efficacy of the two formulations of topical ascorbic acid in reducing the degree and duration of post-CO_2- laser resurfacing erythema (221).

The application of topical L-ascorbic acid in an aqueous formulation resulted in a significant decrease in a post-CO_2-laser resurfacing erythema by the 8th week. The application of topical ascorbic acid in a cream formulation did not give similar results. It was concluded that topical L-ascorbic acid, when used in an appropriate vehicle and when initiated for an appropriate postoperative period, may decrease the degree and duration of erythema after cutaneous CO_2-laser resurfacing (221).

Proliferation of human skin fibroblast was stimulated significantly by the presence of L-ascorbic acid-2-phosphate (Asc 2-P). The presence of Asc 2-P (0.1-1.0 mM) in the culture medium for 3 weeks has increased the relevant ratio of collagen synthesis to total protein synthesis. Supplementation of the medium with Asc 2-P also accelerated procollagen processing to collagen and deposition of collagen in the cell layer. The results indicate that Asc 2-p is useful in culture systems as a long acting vitamin C derivative and also that it promotes reorganization of a three dimensional tissue like substance from skin fibroblasts in culture by stimulating collagen accumulation in the fibroblasts (9).

A study was done to compare 2-o-alpha-D-glucopyranosyl-L-ascorbic acid (AA-2G) with ascorbic acid (AA) and ascorbic acid 2-phosphate (AA-2P) concerning the promotion of collagen production in human skin fibroblasts (222). Although AA-2G was observed to promote collagen synthesis at the same level on the 8th day of the culture, collagen synthesis was seen to decrease on the 5th day of culturing with AA and AA-2P. These findings suggested that AA-2G is decomposed to AA by alpha-glucosidase in the cells. Thus AA promotes collagen synthesis which is prolonged through AA-$2G_s$ sustained decomposition (222).

Human dermal fibroblasts were cultured and aged in vitro. Survivals of young and aged fibroblasts were determined in the presence and absence of different concentrations of two vitamins, vitamin C and vitamin E (223). 5, 12.5, 25, and 50 mumol/L of vitamin C and 1, 5, 10 and 50 mg/L of vitamin E were added 30 minutes before oxidative stress, consisting of exposure to 5 mM hydrogen peroxide for 30 minutes. A non-radioactive cell proliferation cytotoxicity assay (MTT) was used to determine the protective effect of the vitamins studied. Vitamin C produced a clear cytoprotective effect on aged cells

over the entire range of doses applied. The protection provided by vitamin E was also pronounced (223).

Daily use of a topical L-ascorbic acid (vitamin C) product minimizes the appearance of fine lines and wrinkles (224). L-ascorbic acid is an antioxidant which neutralizes the oxygen-free radicals which damage and destroy the skin tissue when stimulated by ultraviolet radiation. Studies also indicate that topical vitamin C offers photoprotective qualities and anti-inflammatory benefits, making it an excellent adjunct to sunscreens. Maintaining healthy skin starts with proper sun protection. Herschthal (224) recommends the daily use of a true broad spectrum sunscreen with a minimum SPF of 20. He claims that the protective products, containing transparent zinc oxide can provide the ultimate in UV protection when used with topical vitamin C. Cautions should be taken since the commercial varieties often contain vitamin C derivatives or insufficient amounts of vitamin C, rendering them ineffective (224).

2.6. Lipacide PVB®

Lipacide® is a condensation product of palmitic acid and hydrolysate of wheat proteins (11). This product has a beige color. Its melting point is 60-70^{0}C. It is soluble in ethanol and oil but very slightly soluble in water (11).

2.6.1. Properties

2.6.1.1. Stimulating Effect on Vital Skin Cells

Cultures of normal human epidermal keratocytes were used as a biological model for the determination of stimulating effect of Lipacide® on the vital skin cells (11). The neosynthesis of proteins was measured by the incorporation of leucine, marked with a radioactive product. This criteria reflects the capacity of keratocytes to synthesize their new proteins. Wheat proteins (Lipacide PVB®) were incubated with normal human epidermal keratocytes for 24 hours. The concentrations selected were in the range of 0.1 – 30 μg/mL, i.e. within the range of solubility of the culture medium. The pH values in the incubation media were maintained at 6.6 and 7.8, respectively. The positive control was composed of an incubation medium containing growth factors. The effect on neosynthesis was assessed using the liquid scintillation technique which measured radioactivity with a PACKARD counter. Results obtained showed that the activity of this

compound is greater in its unchanged form at pH 6.6 than in its ionized form at pH 7.8. It means that the skin's natural acidic pH in the range of 4.2-6.2 promotes the activity of this compound at low concentrations of 0.1% . Two mechanisms could have explained these results (11):

- wheat proteins may have nutritive effect and may provide cells with elements which are absent or insufficient in the culture medium,
- proteins may have a "hormone like" effect acting as a mediator transmitting signals to cells to modify their activity.

Anyhow, it was concluded that wheat proteins have "stimulant" and "trophic" effects on epidermal structures at a low concentration of 0.1% (225).

2.6.1.2. Restructuring Effect on Collagen Fibers

Studies have been carried out on the restructuring effect of wheat proteins on human dermal fibroblasts in cultures (225). This study was based on observation under scanning electron microscope of the network of fibers deposited on the fibroblast culture medium. The method was used to assess semiquantitatively the synthesis of the matrix constituents as well as the quality of the supramolecular arrangement of these molecules. The effects were observed at non-toxic concentrations of wheat protein, i.e. 0.1-0.3 µg/L. Vitamin C known for increasing proline and lysine hydroxylation and thus promoting the formation of cross-links between the matrix's collagen fibers was used as a reference molecule for the experimental validation. Results showed a positive effect on the synthesis and deposit of proteins in the extracellular matrix. These proteins deposited by fibroblasts in the form of fibers on the culture medium mainly contained collagen I, collagen III and some non-collagenic proteins. Morphological and qualitative observations obtained using scanning electron microscope showed a tendency towards fiber elongation in the presence of wheat proteins as for vitamin C. This effect seemed to be dose dependent and more severe at pH 6.6 than at pH 7.8. Consequently, wheat proteins are active contituents which contribute to cutaneous restruction and firmness because of their effect on the synthesis and the organization of the skin matrix.

2.6.1.3. Moisturizing Effect

The moisturizing potentials of wheat proteins were studied using the traditional "gelatin cells method", at two concentrations of 2 and 5 % in a cream (25). This method was used to determine the *in-vitro* effect of a product against transepidermal water loss (226). The results showed that when the proteins were incorporated at concentrations of 2 and 5 %, they limited transepidermal water loss (TEWL) thus promoting skin moisturization.

2.6.1.4. Toxicology of Wheat Proteins

When tested in a 5% dilution in vaseline in a patch test, these proteins showed an acceptable level of tolerance (227). Therefore, these proteins can be incorporated into any formulation upto a concentration of 5 % without any toxic or side effects.

3. MATERIALS AND METHODS

3.1. Materials and Apparatus

3.1.1. Chemicals

Abil EM 90	Goldschmidt, Germany
Immersion oil	Olympus Optical Co. Ltd., Japan
L(+)Ascorbic acid	E. Merck, Germany
Lipacid PVB	Givaudan-Lavirotte, France
Macadamia nut oil	Alban Müller International, France
Magnesium sulfate	E. Merck, Germany
Nile red	Sigma, Germany
Synperonic PE/F 127	Uniqema, Belgium
Triethanolamine	Carlo Erba, Italy

3.1.2. Apparatus And Software

Balance	Scaltec SBA, 31
Camera	Olympus attached to microscope
Centrifugator	Hettich EBA-8
Cutometer	Courage & Khazaka
Globule Size Analyser	Malvern, Mastersizer 2000
Mechanical Stirrer	Heidolph RZR 2101
Mexameter	Courage & Khazaka
Microscope	Olympus Bx50
Oven	Elektro-Mag
pH-Meter	WTW
Refrigerator	Arçelik
Rheometer	Brookfield DV III
Sebumeter	Courage & Khazaka
Skin-pH meter	Courage & Khazaka
SPSS	10.0, 2000
Stability Cabinet	Aymes
Water Bath	Polyscience CFC Refregerent
Water Bath	GFL 1052

3.2. METHODS

3.2.1. Basic Properties of Macadamia Nut Oil

Cosmetic oils, like all cosmetic ingredients, must be subjected to a comprehensive evaluation. The evaluation criteria includes the effect of physico-chemical properties on the colloid chemistry and the sensory properties of emulsions. Five basic properties are defined which influence the colloid chemistry as well as sensory characteristics of emulsion preparations (228). Basic properties, namely, viscosity, surface tension, pour and cloud points, spreadability and polarity are parameters used to define cosmetic oils (229).

3.2.1.1. Rheological Analyses

The rheological test was performed using a thermo-controlled stress rheometer in the cone and plate geometry.

3.2.1.2. Surface Tension

Capillary rise method at 25±1^{0}C was used to determine the surface tension between macadamia nut oil and water. Diameter of the capillary was measured using a compass and following the determination of densities of the oil and water, surface tension was calculated. Each measurement was repeated 5 times and the mean was taken.

3.2.1.3. Spreadability

The method proposed by Roehl and Brand (232) was used to test the spreadability of the oil using glass plates coated with gelatin. 1 % aqueous solution of gelatin was prepared by gentle heating. Microscopic slides which have a flat, glass surface were wetted uniformly with the gelatin solution. The film was allowed to solidify on a tray floating on an ice bath for 1 hour. The film was then dried at room temperature for two days, followed by storage at 25^{0}C, dust-free cabin for 24 hour. The spreadability tests were performed in a conditioned room of 52 % relative humidity and 23^{0}C temperature. Coated glass plates were placed on a milimeter graph paper on a flat surface. 10 µL of the oil to be tested was put on a plate using a microliter pipette. After 5 minutes, the diameter of the circular spreading area was read. The test was repeated 5 times and the mean measurements were assigned to the given standards (228).

3.2.1.4. Pour Point and Cloud Point

The determination of pour and cloud points of macadamia oil was performed in a water bath (± 0.1^{0}C). 15 mL of oil was put into the test tube which was placed in the water bath. The temperature of the water bath was decreased gradually and the point where clouding appeared was noted. Again, the temperature of the water bath was decreased and the point at which the oil solidified was noted. Each test was repeated three times.

3.2.1.5. Polarity

The method proposed recently to set a scale of polarity for cosmetic oils with the help of the solvatochrome dye, nile red, was used to determine the polarity of oil and the mixture of oil and lipophilic surfactant (229). The test was repeated thrice for each.

12 mg of nile red was weighed into a 10 mL volumetric flask and made up with chloroform. From this stock solution, 50 µL was transferred into a 10 mL volumetric flask and chloroform was evaporated ensuring the even distribution of the dye on the glass wall. The sample to be tested was then filled up to 10 mL and agitated for 12 hours using a small magnetic bar, until the dye has dissolved completely. UV spectrum was determined with UV-photospectrometer (Shimadzu UV-160 A) with the oil and the mixture as reference.

3.2.2. Preparation of Multiple Emulsion

In this study, multiple emulsions were prepared by the two step process (79, 80).

3.2.2.1. Two step Process

3.2.2.1.1. Preparation of the Base

Primary emulsion was prepared by heating oil phase consisting of macadamia nut oil and lipophilic surfactant (Abil EM 90) to 75^{0}C±1^{0}C. Aqueous phase consisting of water and magnesium sulphate was also heated to the same temperature. Aqueous phase was added to the oil phase drop by drop while stirring at 2000 rpm. Agitation was continued until cooling to room temperature of 25^{0}C.

For the multiple emulsion, aqueous phase consisted of water and the hydrophilic surfactant (Synperonic PEF/127). Primary emulsion was added little by little to the aqueous phase at 1000 rpm for 10 minutes. Emulsion was then homogenized at 700 rpm for 7 minutes.

3.2.2.1.2. Preparation of the Active Formulation

Oil phase which consisted of macadamia nut oil and surfactant (Abil EM 90), was heated upto $75^0C \pm 1^0C$. Lipacide was added when the temperature was acquired. At the same time, aqueous phase consisting of water and magnesium sulphate was heated to the same temperature. Vitamin C was added at last to the aqueous phase. Aqueous phase was added to the oil phase drop by drop. Stirring was continued at 2000 rpm by the microvortex mixer, until the emulsion cooled to room temperature, for about 25 minutes.

In the next step, multiple emulsion was prepared. Aqueous phase consisted of Synperonic, the hydrophilic emulsifier, and water. Triethanolamine was added to adjust the pH of the emulsion. Primary emulsion was added little by little to the aqueous phase at 700 rpm in 20 minutes. After the complete addition of the PE, the speed of the mixer was reduced to 400 rpm for homogenization, for a period of 35 minutes.

Formula of Base

Primary Emulsion

Macadamia nut oil	26%
Abil EM 90	4%
Magnesium sulfate	0.7%
Distilled Water q.s.	100%

Multiple Emulsion

P.E.	80%
Synperonic	0.8%
Distilled Water q.s.	100%

Formula of Active Formulation

Primary Emulsion

Macadamia nut oil	26%
Abil EM 90	6%
Lipacide PVB	0.5%
Magnesium sulfate	0.7%
Ascorbic acid	1.0%
Distilled Water q.s.	100%

Multiple Emulsion

PE	75%
Symperonic PEF/127	2.0%
Triethanolamine	0.7%
Distilled Water q.s.	100%

3.2.3. Properties of Primary and Multiple Emulsions

Primary and multiple emulsions were analysed to assure the formation of the desired emulsions.

3.2.3.1. Physical Analysis

Primary and multiple emulsions were analysed organoleptically (color, thickness, look, feel) and physically (creaming and phase separation).

3.2.3.1.1. Types of Emulsions

Types of emulsions were determined by diluting the emulsion with oil and water separately.

3.2.3.1.2. Microscopic Test

Multiple emulsions were analysed under microscope to confirm the multiple character. A drop of multiple emulsion was placed on the glass slide and diluted with water homogenously and covered by glass slide cover. A drop of immersion oil was placed on the cover slide and observed under the microscope (110).

3.2.3.1.3. Globule Size

Globule sizes of the multiple emulsions were determined for the freshly prepared emulsions and for the emulsions kept at different conditions, i.e. $4^0C\pm0.1^0C$, $25^0C\pm0.1^0C$, $40^0C\pm0.1^0C$ and $40^0C\pm0.1^0C$ with 60% relative humidity (RH) conditions. Analyses were performed after every 15 days upto 6 months. Globule sizes were determined by an instrument utilizing laser light diffraction phenomenon (112).

3.2.3.1.4. pH Determination

pH values of the freshly prepared emulsions and the emulsions kept at different conditions were determined by a digital pH-Meter.

3.2.3.1.5. Centrifugation Test

Centrifugal tests were performed for primary emulsions and for multiple emulsions immediately after preparation. The centrifugal tests were repeated for multiple emulsions after 24 hours of preparation. These tests were performed at 25^0C and at 5000 rpm for 5 minutes by placing 10 g of samples in the centrifugal tubes.

3.2.3.1.6. Stability Tests

Stability tests were performed at different temperatures and different conditions for multiple emulsions to see the effect of these conditions on the storage of multiple emulsions. These tests were performed on samples kept at $4^0C\pm0.1^0C$ (in refrigerator), $25^0C\pm0.1^0C$ (in oven), $40^0C\pm0.1^0C$ (in oven) and at $40^0C\pm0.1^0C$ with 60% RH (in cabinet). Physical characteristics of multiple emulsions, i.e. color, creaming, liquifaction, pH and globule sizes were noted at various intervals for 6 months.

3.2.3.1.7. Rheological Tests

Rheological tests were performed for freshly prepared multiple emulsions and multiple emulsions stored at different conditions in order to see any changes produced by these conditions. For this purpose, programmable cone-plate (Brookfield) rheometer was used. The cone used had an angle of 1.565^0, radius of 1.2 cm and a shear rate of 3.84XN (N=rpm). Approximately 0.2 g samples and a

1.2 cm and a shear rate of 3.84XN (N=rpm). Approximately 0.2 g samples and a constant temperature of 25^{0}C were used for the tests. Tests were repeated three times, each containing 9 values of shear rate.

Following the determination of the flow type, flow curves were fit to one of the available mathematical models. These tests were performed at 25^{0}C by using 0.2 g of multiple emulsions. Increased shear stresses were applied on the samples and the shear rates and changes in viscosities were noted.

3.2.4. Product Evaluation on Skin

11 volunteers were selected whose ages were in between 40 and 65 years. Male and female volunteers were included in this work. Prior to the tests, the volunteers were examined by a cosmetic expert for any serious skin disease or damage especially on cheeks and forearms. Before the study, every volunteer was provided with a volunteer protocol **(Figure 3.1.)**. This protocol stating the terms and conditions of the testing was signed by every volunteer individually. Volunteers were not informed about the contents of formulations. All the skin tests were done at 25^{0}C and 40% relative humidity conditions. On the first day, patch test (Burchard test) was performed on the forearms of each volunteer to determine any possible reactions to the multiple emulsions. In addition, basic values of skin moisture, sebum, pH and elasticity were noted for the right and left cheeks of each individual. On the second day, each volunteer was provided with two creams. One cream was base and the other one was formulation containing the active ingredients. Each cream was marked with "right" or "left" indicating application of that cream to the respective cheek. The creams were applied by the volunteers themselves as instructed for 28 days. Every individual was instructed to come on days 8, 15, 22 and 29 for the skin measurements.

Type of Preparation

Facial cream

Duration of study

4 weeks

Daily use

Twice a day, morning and evening

Region of Application

Cheeks

Tests to Perform

-Burchard test

-Mexameter

-Corneometer

-Sebumeter

-Skin pH meter

-Cutometer

-Panel test

Essential criteria for being included in the study

1) Individuals should be between 40-65 years of age, healthy male or female.
2) No person should be hypersensitive to creams. There should be no lesions, diseases or any abnormality on the skin of face and forearm.
3) No body should use any other cream or any other product on the face, at least 3 days before the study.
4) Every individual should read and sign the volunteer protocol.
5) During the study, every individual should come to the laboratory for measurements.

Criteria for Excluding Volunteers from the study

6) Pregnant women or milk feeding women.
7) Any individual under treatment for skin disorders during the study.
8) Any individual hypersensitive to any ingredient of the cream or with history of sensitivity.
9) Any woman taking estrogen, progesteron or any other oral contraceptive at least 12 weeks before the study.
10) Any individual who does not obey the application rules of the creams.
11) Any individual with extraordinary hair on the cheeks.

No body can insist to be informed about the composition of creams nor about the results of the study.

Volunteer	Supervisor of the study
Name	Name
Age	Signature
Signature	

Figure 3.1. Volunteer Protocol

3.2.4.1. Patch Tests (Burchard Tests)

On the first day of skin testing, patch tests were performed on the forearms of each volunteer. A 5 cmX4 cm regions were marked on the forearms. Basic values for erythema and melanin were measured with the help of Mexameter. 1.0 g of base and formulation each were applied to the 5 cmX4 cm marked regions separately on each forearm. The regions were covered with the surgical dressing after application. After 24 hours, dressings were removed and the measurements of erythema and melanin were repeated on both forearms.

3.2.4.2. Mexameter

Mexameter was used to measure erythema and melanin on the cheeks, on the first day before the application of any cream and then on days 8, 15, 22 and 29.

3.2.4.3. Corneometer

With the help of a Corneometer, moisture of the skin was measured before the application of any cream and then on days 8, 15, 22 and 29.

3.2.4.4. Sebumeter

Sebumeter was used to measure sebum content of the skin before application of any cream and then on days 8, 15, 22 and 29.

3.2.4.5. Skin pH-meter

pH value of the skin was measured with the help of a skin pH-Meter before application of creams and then on days 8, 15, 22 and 29.

3.2.4.6. Cutometer

Net elasticity of the skin was measured by the aid of a Cutometer before application of creams and then on days 8, 15, 22 and 29.

3.2.4.7. Panel test

Every individual was provided with a form prepared previously to test the sensory values of both creams. This form consisted of seven parameters to be evaluated and every parameter was assigned 11 values from –5 to +5 indicating very bad to very good, respectively **(Figure 3.2.)**. This form was asked to be completed independently by each individual on day 29.

PANEL TEST

1. Ease of application
2. Spreadability
3. Sense just after application
4. Sense in long-term
5. Irritation
6. Shine on skin
7. Sense of softness

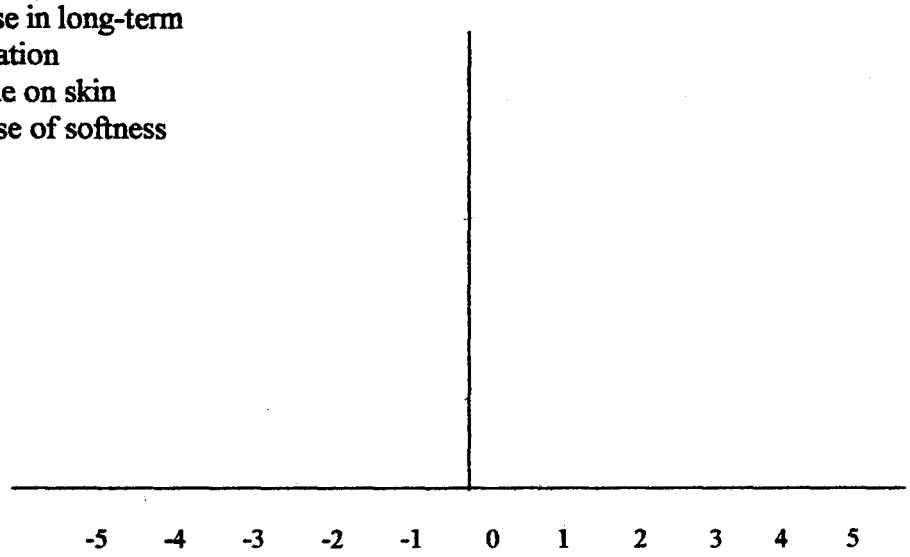


Figure 3.2. Form of the Panel Test

3.2.4.8. Statistical Analyses

The measured values obtained for different parameters (skin moisture, sebum, melanin, erythema, elasticity and pH) were analysed using SPSS 10.0 on the PC computer (paired samples t-test for variation between the two preparations; two-way ANOVA for variation between different time intervals).

4. RESULTS and DISCUSSION

4.1. RESULTS

4.1.1. Basic Properties of Macadamia Nut Oil

Basic properties of macadamia nut oil have been given in **Table 4.1.** and the rheogram has been shown in **Figure 4.1.**

Table 4.1. Basic Properties of Macadamia Nut Oil

Viscosity ±SE mPas.S (n=10)	Surface Tension±SE mN/m (n=5)	Spreadability ±SE (mm) (n=5)	Solvatochromism (nm) (n=3) ±SE Mixture		Pour Point (°C) ±SE (n=3)	Cloud Point (°C) ±SE (n=3)
			Oil	Mixture		
40.42 ± 0.77	33.03 ± 0.33	8.2 ± 0.02	525.5 ± 0.29	518.3 ± 4.94	-1.83 ± 0.17	0.17± 0.17

SE= Standard Error

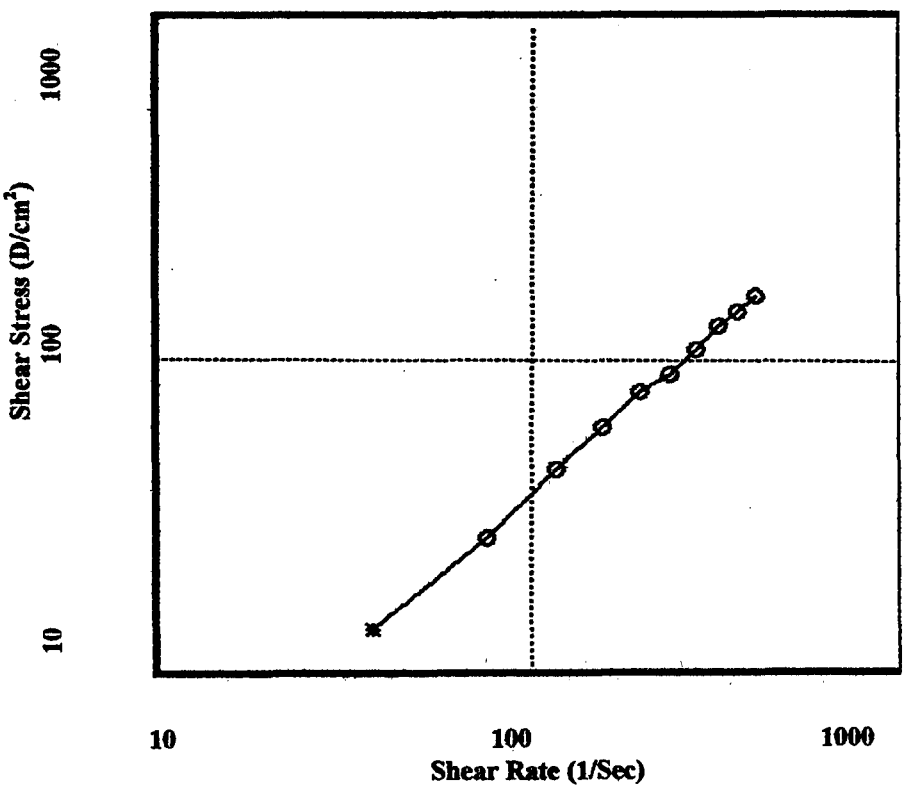


Figure 4.1. Rheogram of Macadamia Nut Oil

4.1.2. Globule Sizes

Globule sizes of the formulations kept at different conditions upto six months have been examined and given in **Table 4.2.** and represented in **Figures 4.2.-4.5.**

Table 4.2. Globule Sizes ±Standard Errors of Base (B) and Active Formulation (F) Kept at Different Conditions (n=3)

	Fresh		After 15 Days		After 1 Month		After 45 Days		After 2 Months		After 3 Months		After 6 Months	
	B	F	B	F	B	F	B	F	B	F	B	F	B	F
A	12.29 ± 0.46	5.43 ± 0.04	-	-	-	-	-	-	15.70 ± 0.41	4.65 ± 0.05	14.98 ± 0.23	6.69 ± 0.25	18.92 ± 0.73	7.37 ± 0.16
B	12.29 ± 0.46	5.43 ± 0.04	-	-	-	-	-	-	14.38 ± 0.28	4.51 ± 0.06	14.18 ± 0.55	5.03 ± 0.02	14.76 ± 0.48	5.10 ± 0.05
C	12.29 ± 0.46	5.43 ± 0.04	-	-	-	-	-	-	13.51 ± 0.42	4.16 ± 0.13	16.16 ± 0.22	6.22 ± 0.03	13.7 ± 0.34	6.34 ± 0.24
D	12.29 ± 0.46	5.43 ± 0.04	12.59 ± 0.19	4.42 ± 0.10	11.74 ± 0.39	4.41 ± 0.14	15.16 ± 0.29	4.19 ± 0.10	13.80 ± 0.33	4.21 ± 0.03	14.52 ± 0.56	6.53 ± 0.08	14.59 ± 0.56	6.53 ± 0.18

A = 4°C; B = 25°C; C = 40°C; D = 40°C & 60 % RH

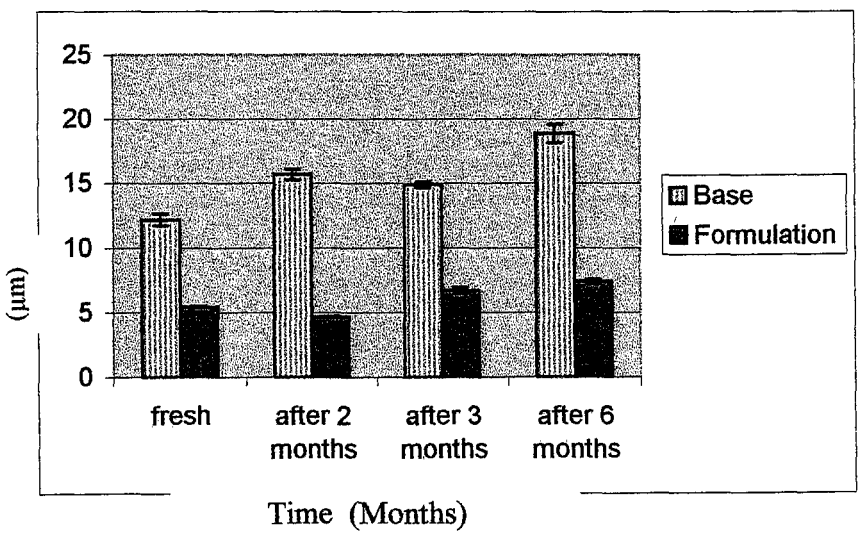


Figure 4.2. Globule Sizes of Base and Formulation Kept at 4°C

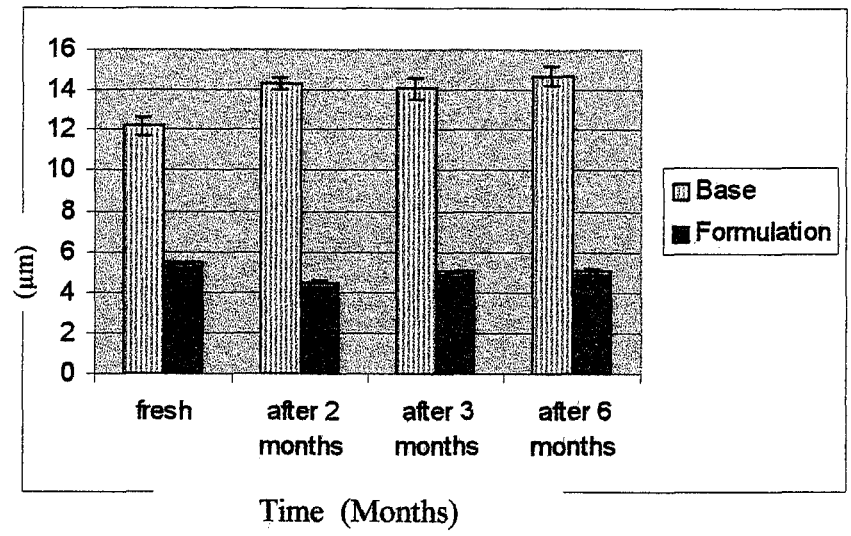


Figure 4.3. Globule Sizes of Base and Formulation Kept at 25°C

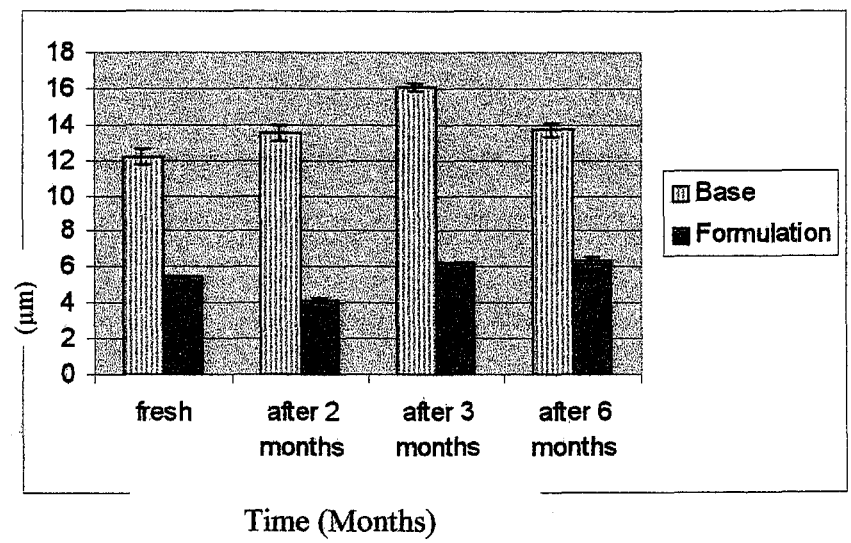


Figure 4.4. Globule Sizes of Base and Formulation Kept at 40°C

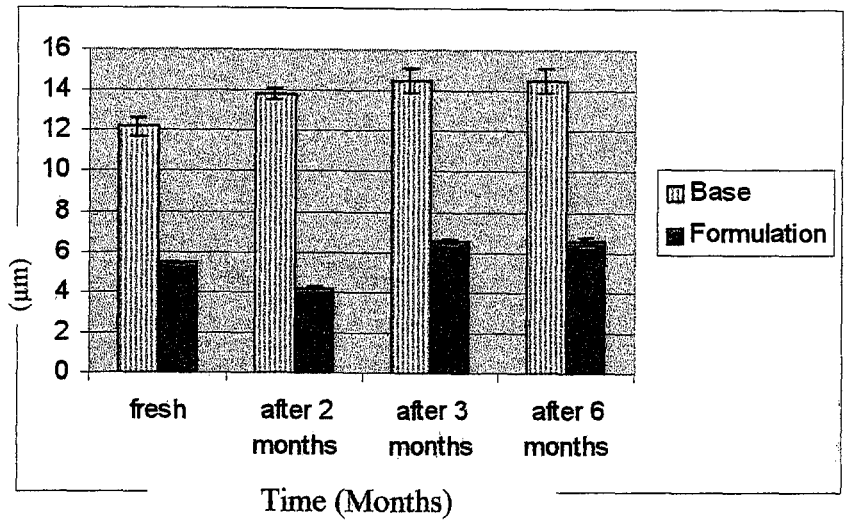


Figure 4.5. Globule Sizes of Base and Formulation Kept at 40°C & 60 % RH

4.1.3. pH

pH values of the formulations kept at different conditions upto six months have been determined and represented in **Table 4.3.** pH values of base and formulations have been shown in **Figures 4.6. - 4.9.**

Table 4.3. pH Values ± Standard Errors of Base (B) and Active Formulation (F) Kept at Different Conditions (n=3)

	Fresh		After 15 Days		After 1 Month		After 45 Days		After 2 Months		After 3 Months		After 6 Months	
	B	**F**	**B**	**F**	**B**	**F**	**B**	**F**	**B**	**F**	**B**	**F**	**B**	**F**
A	6.64 ± 0.0	6.27 ± 0.0	-	-	-	-	-	-	5.69 ± 0.01	4.81 ± 0.00	5.54 ± 0.03	4.28 ± 0.00	4.64 ± 0.02	4.36 ± 0.01
B	6.64 ± 0.0	6.27 ± 0.0	-	-	-	-	-	-	5.67 ± 0.05	5.21 ± 0.10	5.45 ± 0.02	4.78 ± 0.00	5.49 ± 0.01	4.77 ± 0.01
C	6.64 ± 0.0	6.27 ± 0.0	-	-	-	-	-	-	4.02 ± 0.18	5.13 ± 0.00	3.44 ± 0.04	4.60 ± 0.0	3.31 ± 0.04	4.59 ± 0.01
D	6.64 ± 0.0	6.27 ± 0.0	6.46 ± 0.02	5.46 ± 0.03	6.37 ± 0.05	5.30 ± 0.01	6.14 ± 0.00	5.05 ± 0.00	6.17 ± 0.00	4.89 ± 0.01	4.76 ± 0.00	4.53 ± 0.00	3.79 ± 0.02	4.53 ± 0.01

A = 4°C; B = 25°C; C = 40°C; D = 40°C & 60 % RH

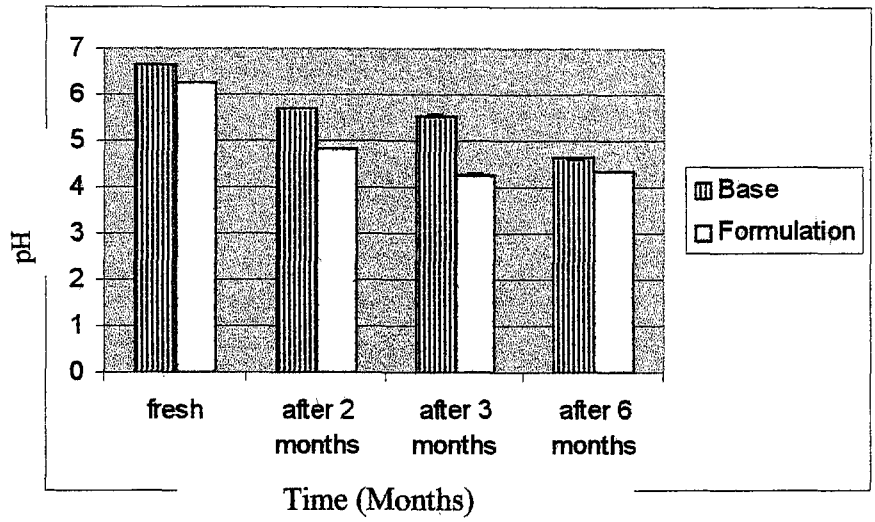


Figure 4.6. pH Values of Base and Formulation Kept at 4°C

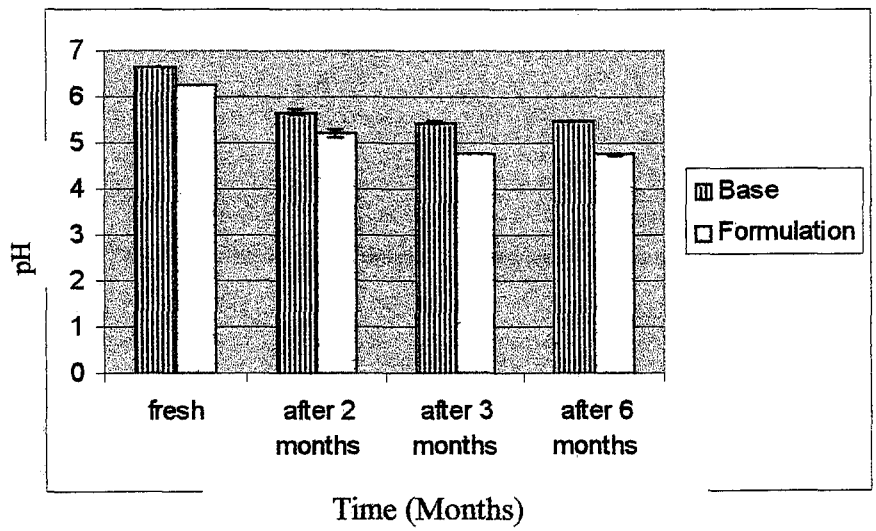


Figure 4.7. pH Values of Base and Formulation Kept at 25°C

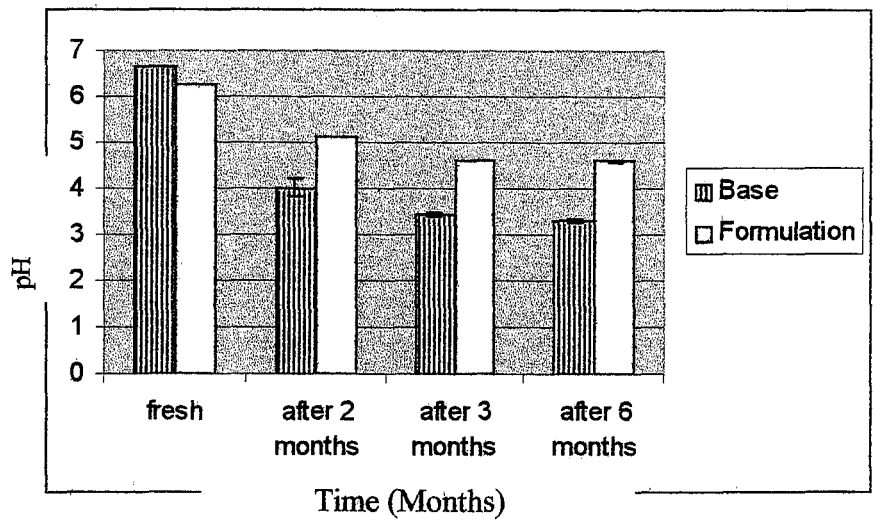


Figure 4.8. pH Values of Base and Formulation Kept at 40^0C

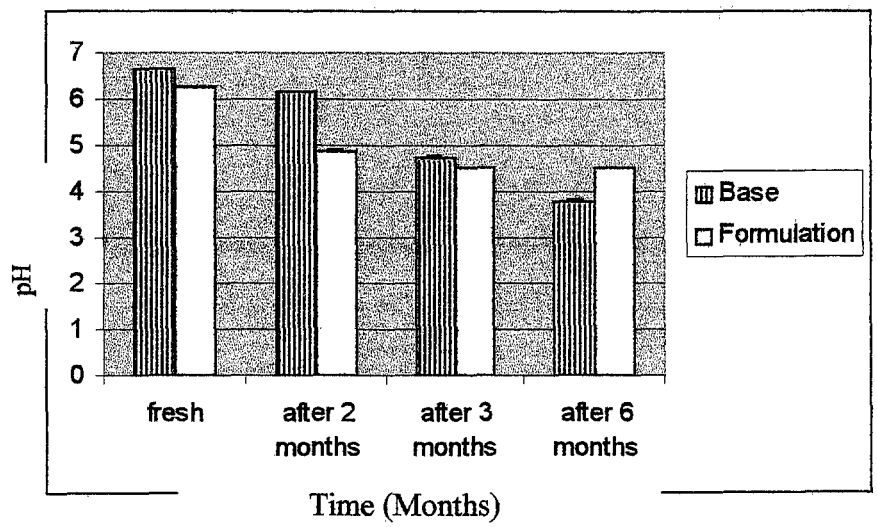


Figure 4.9. pH Values of Base and Formulation Kept at 40^0C & 60 % RH

4.1.4. Stability

Stabilities of base and active formulation kept at different storage conditions were studied. The physical characteristics regarding the stabilities of base and formulation have been presented in **Table 4.4.** Photographs of freshly prepared base and formulation and formulations kept at different conditions for three months were taken and have been given in **Figures 4.10. - 4.16.**

Table 4.4. Physical Characteristics of Base (B) and Formulation (F) Kept at Different Conditions.

		Fresh		After 15 Days		After 1 Month		After 45 Days		After 2 Months		After 3 Months		After 6 Months	
		B	F	B	F	B	F	B	F	B	F	B	F	B	F
Color	A	W	W	W	W	W	W	W	W	W	YW	W	YW	W	YW
	B	W	W	W	W	W	W	W	YW	W	YW	W	YW	W	YW
	C	W	W	W	YW	W	YW	W	YW	W	YW	W	YW	W	YW
	D	W	W	W	YW	W	YW	W	YW	W	YW	W	YW	W	YW
Liquifaction	A	No	No	No	No	No	No	No	+	No	+	No	++	No	++
	B	No	No	No	No	No	No	No	+	No	+	No	+	No	+
	C	No	No	No	+	No	+	No	+	No	++	No	++	No	++
	D	No	No	No	+	No	+	No	+	No	++	No	++	No	++
Phase Separation	A	No	No	No	No	No	No	No	No	No	No	No	No	No	No
	B	No	No	No	No	No	No	No	No	No	No	No	No	No	No
	C	No	No	No	No	No	No	No	No	No	No	No	No	No	No
	D	No	No	No	No	No	No	No	No	No	No	No	No	No	No

W=White; YW=Yellowish White; + = Little; ++ = More; A=At 4^0C; B=At 25^0C; C= At 40^0C; D= At 40^0C & 60% RH.

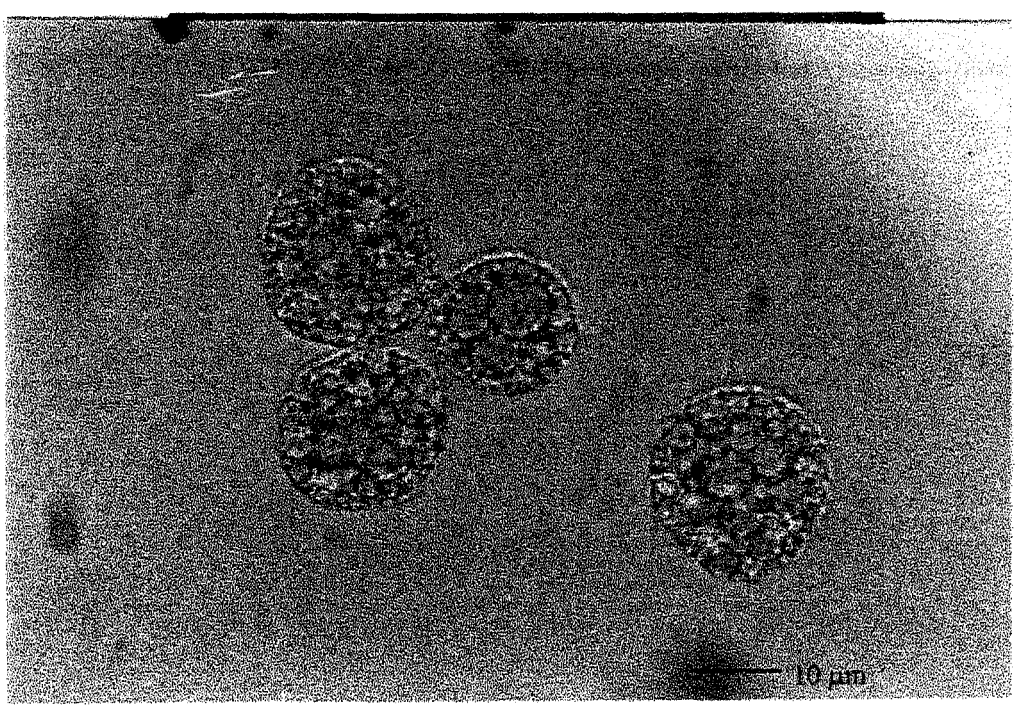


Figure 4.10. Freshly Prepared Base

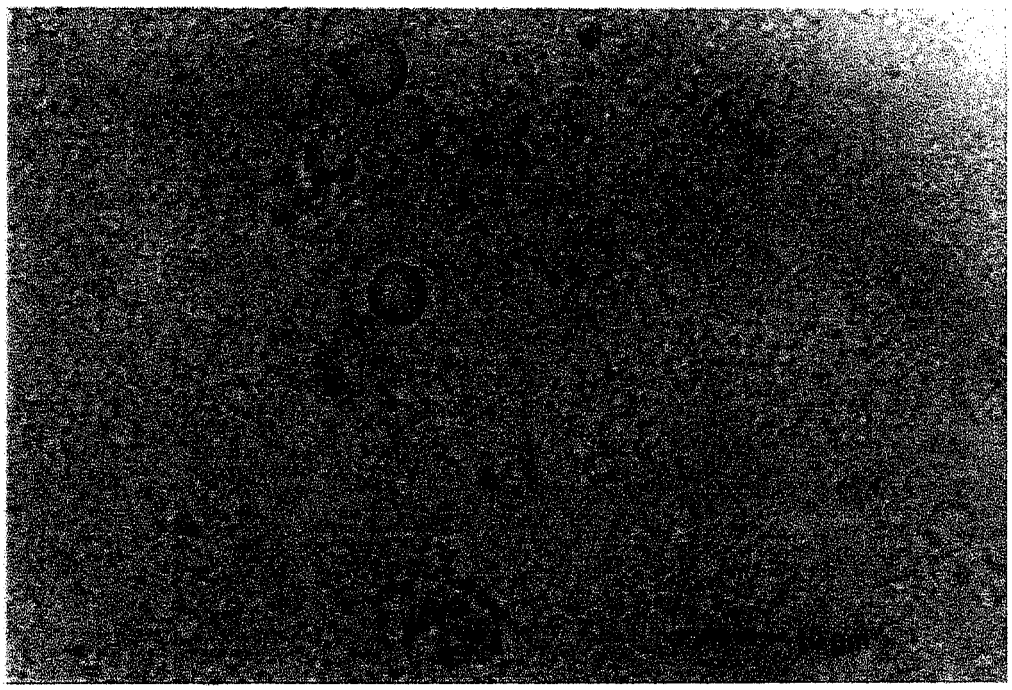


Figure 4.11. Base Kept at 25^0C for 3 Months

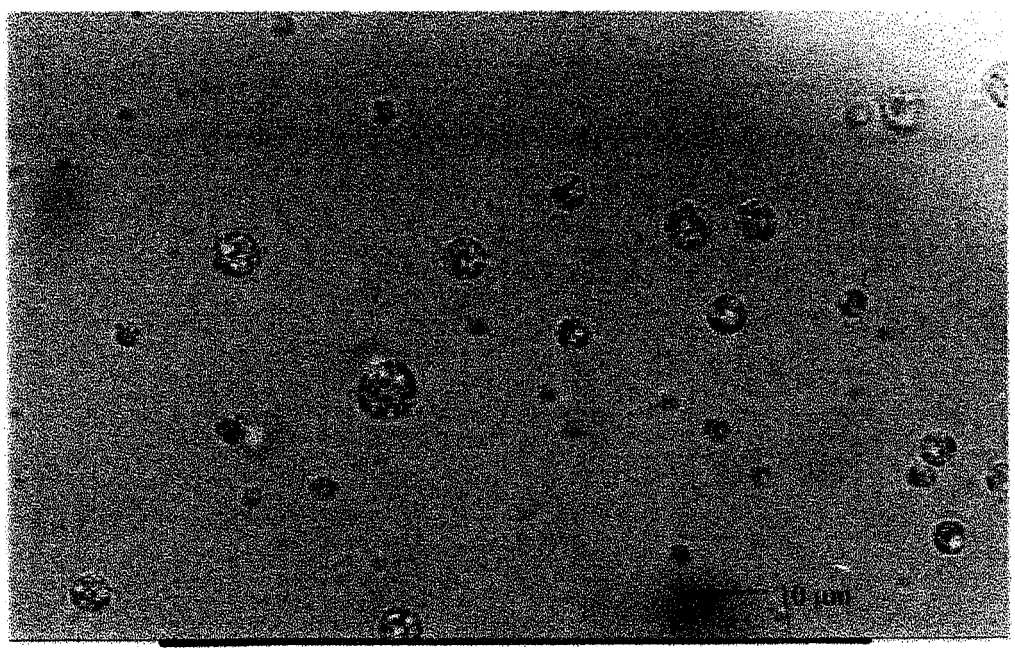


Figure 4.12. Freshly Prepared Formulation

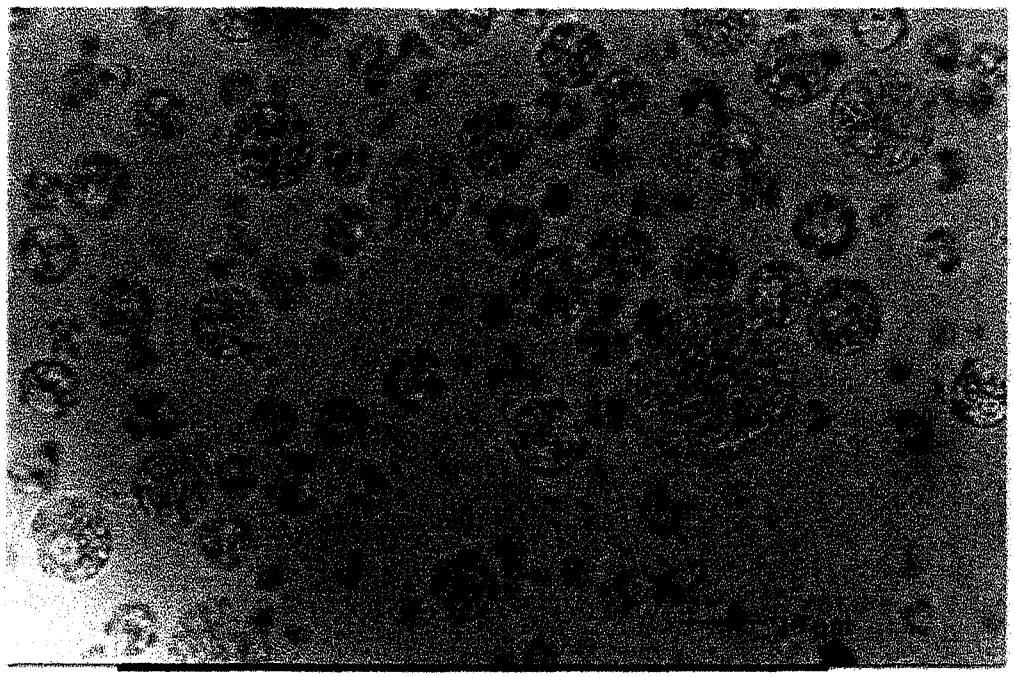


Figure 4.13. Formulation Kept at 4^0C for 3 Months

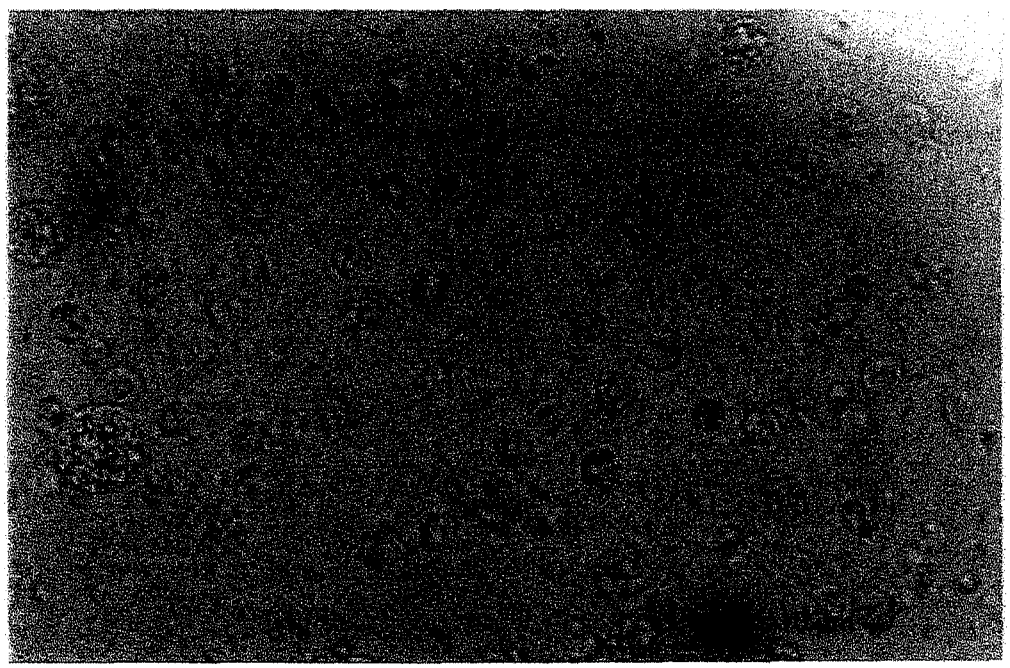


Figure 4.14. Formulation Kept at 25^0C for 3 Months

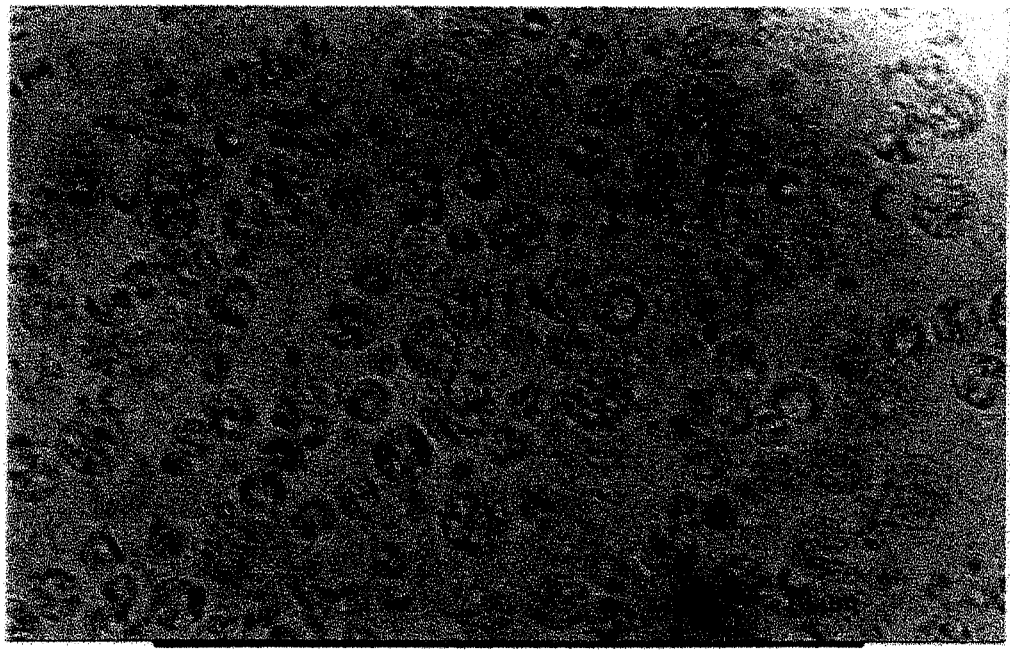


Figure 4.15. Formulation Kept at 40^0C for 3 Months

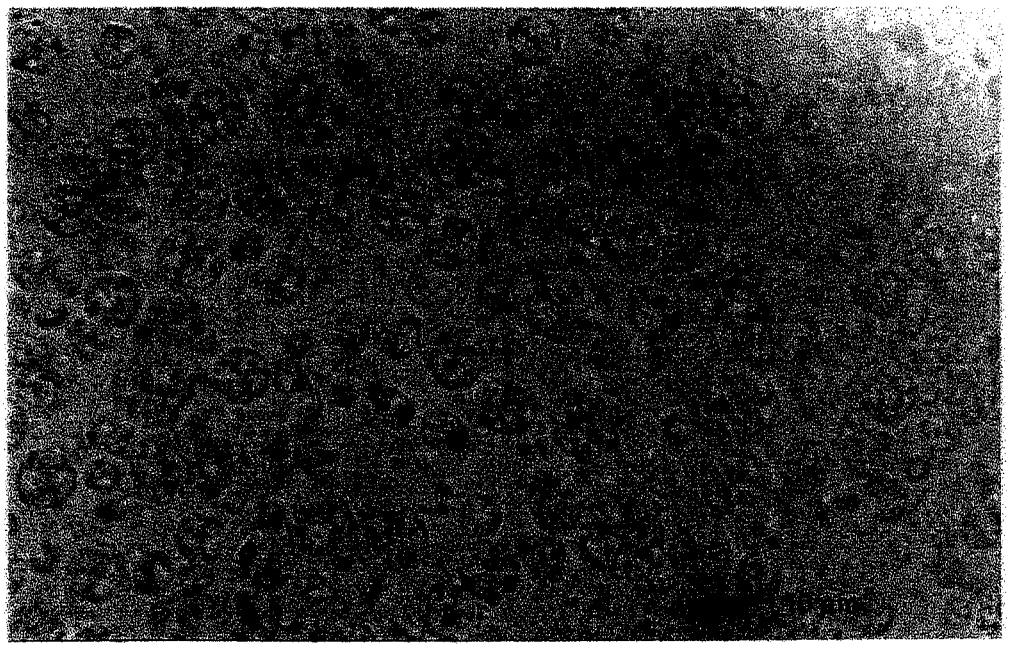


Figure 4.16. Formulation Kept at 40^0C & 60 % RH for 3 Months

4.1.5. Rheological Studies

Rheological properties of formulations kept at different conditions upto six months were noted and have been given in **Table 4.5.** Rheograms of the formulations have been demonstrated in **Figure 4.17. - 4.34.** Rheological parameters of the formulations are presented in **Table 4.6.**

Table 4.5. Viscosities (mPas.s) ± Standard Errors of Base (B) and Active Formulation (F) Kept at Different Conditions

Parameters			4°C				25°C				40°C				40°C & 60 % RH			
	B	F	B		F		B		F		B		F		B		F	
	Fresh	Fresh	3m	6m	3m	6m	3m	6m	3m	6m	3m	6m	3m	6m	3m	6m	3m	6m
1	28501.3 ±1194.7	10581.3 ±451.6	23552.0 ±1288.5	26282.0 ±1682.7	5632.0 ±0.0	5888.0 ±256.0	16213.3 ±682.7	24576.0 ±1182.5	14848.0 ±1024.2	26624.0 ±782.1	19797.3 ±451.6	27818.7 ±615.4	11605.3 ±1194.7	46592.0 ±2048.3	19797.3 ±170.7	36181.3 ±1732.1	13653.3 ±1397.0	24832.0 ±22275.0
2	22101.3 ±961.7	9301.3 ±307.7	16298.7 ±1230.7	18005.3 ±890.9	4096.0 ±0.0	4352.0 ±256.0	12544.0 ±512.0	21589.3 ±615.4	10112.0 ±896.1	18432.0 ±532.9	18602.7 ±85.3	21162.7 ±840.5	8448.0 ±823.0	46336.0 ±4864.7	16384.0 ±147.8	28160.0 ±591.2	9301.3 ±341.3	31744.0 ±30212.0
3	18432.0 ±599.6	8419.7 ±300.1	12913.7 ±692.0	14393.0 ±593.9	3413.5 ±170.5	3584.0 ±341.1	10695.0 ±316.8	18887.0 ±542.8	8277.0 ±256.0	14734.3 ±316.7	16441.0 ±57.0	16896.0 ±806.6	7111.0 ±594.0	39850.5 ±938.6	14222.3 ±316.7	21959.0 ±247.9	7566.0 ±346.2	24661.0 ±23299.0
4	16256.0 ±391.1	7638.3 ±238.2	11050.7 ±503.1	12416.0 ±461.5	2944.0 ±128.0	2624.0 ±320.1	9685.3 ±379.2	16682.7 ±364.6	7040.0 ±256.0	12586.7 ±170.7	14508.0 ±278.5	14634.7 ±480.8	6442.7 ±186.0	32896.0 ±0.0	12672.0 ±73.9	18645.3 ±298.7	6442.7 ±237.6	21440.0 ±20675.0
5	14336.0 ±359.6	7202.3 ±279.5	9796.3 ±436.0	11262.0 ±354.5	2611.0 ±51.0	2201.5 ±153.5	8772.3 ±291.7	15223.3 ±435.9	6348.5 ±102.5	11195.7 ±223.8	13074.3 ±89.3	12834.3 ±384.6	5768.7 ±136.7	28100.3 ±2940.7	11400.3 ±34.3	16213.3 ±90.3	5734.3 ±177.5	20958.0 ±12334.0
6	13539.7 ±356.5	6855.3 ±297.0	8960.0 ±421.0	10382± 382.6	2389.5 ±170.5	2176.0 ±128.0	8277.3 ±260.9	14080.0 ±299.5	5675.0 ±128.0	10154.7 ±49.4	12003.7 ±102.7	11434.7 ±177.6	5063.0 ±75.2	20949.5 ±213.5	10666.7 ±85.3	14734.0 ±199.2	5120.0 ±130.4	14549.0 ±13613.0
7	12726.7 ±295.7	6607.3 ±254.6	8289.7 ±425.2	9825.3± 281.2	2157.5 ±36.5	1938.5 ±109.5	7850.7 ±240.3	12970.7 ±208.2	5156.5 ±109.5	9289.0 ±42.2	11093.3 ±64.7	10459.3 ±263.5	4754.3 ±84.6	19273.0 ±110.0	9996.0 ±49.0	13653.7 ±24.3	4729.7 ±48.7	12507.0 ±11778.0
8	12138.7 ±237.6	6293.3 ±213.3	7893.3 ±408.7	9386.7 ±377.4	2016.0 ±160.0	1792.0 ±64.0	7509.3 ±240.4	12074.7 ±139.9	4736.0 ±64.0	8554.7 ±76.9	10368.0 ±64.0	9706.7 ±186.0	4224.0 ±37.0	17472.0 ±320.1	9493.3 ±85.3	12928.0 ±66.1	4352.0 ±37.0	10144.0 ±9441.4
9	11529.7 ±218.8	6030.7 ±170.7	7490.0 ±312.2	8855.7 ±366.31	1906.0 ±142.0	1678.5 ±28.5	7300.7 ±255.3	11263.7 ±143.1	4438.0 ±56.0	7983.7 ±162.0	9652.3 ±105.5	8912.7 ±148.1	3906.0 ±38.0	15673.0 ±199.0	9007.7 ±132.7	12345.0 ±57.0	3925.0 ±32.9	8874.5.0 ±8193.7
10	11093.3 ±178.4	5836.7 ±153.7	7167.8 ±241.9	8362.7 ±246.1	1843.5 ±102.5	1459.0 ±77.0	7048.7 ±238.7	10735.0 ±103.9	4173.0 ±77.0	7697.0 ±104.0	9079.3 ±45.3	8209.0 ±151.7	3669.3 ±45.2	35354.0 ±1408.2	8618.7 ±173.2	11673.7 ±51.3	3686.0 ±0.0	15718.0 ±150.5

3 m = 3 months; 6 m = 6 months

Table 4.6. Different Rheological Parameters of Base (B) and Active Formulation (F)

Parameters			4°C				25°C				40°C				40°C & 60 % RH			
	B	F	B		F		B		F		B		F		B		F	
	Fresh	Fresh	3 m	6 m	3 m	6 m	3 m	6 m	3 m	6 m	3 m	6 m	3 m	6 m	3 m	6 m	3 m	6 m
ST	1.69 ± 0.03	1.35 ± 0.02	2.07 ± 0.06	1.99 ± 0.06	1.95 ± 0.09	2.54 ± 0.03	1.69 ± 0.03	1.56 ± 0.04	2.03 ± 0.11	2.17 ± 0.04	1.51 ± 0.03	2.13 ± 0.08	2.02 ± 0.19	1.14 ± 0.01	1.57 ± 0.03	2.06 ± 0.07	2.35 ± 0.25	1.82 ± 0.59
Thix Index	2.57 ± 0.07	1.81 ± 0.05	3.28 ± 0.11	3.13 ± 0.11	3.07 ± 0.18	4.04 ± 0.04	2.57 ± 0.07	2.29 ± 0.09	3.56 ± 0.18	3.46 ± 0.08	2.18 ± 0.06	3.39 ± 0.14	3.16 ± 0.33	1.32 ± 0.01	2.30 ± 0.07	3.28 ± 0.14	3.70 ± 0.38	2.69 ± 1.16
Flow Index	0.58 ± 0.01	0.74 ± 0.01	0.48 ± 0.01	0.51 ± 0.01	0.50 ± 0.03	0.39 ± 0.0	0.58 ± 0.01	0.63 ± 0.02	0.45 ± 0.03	0.44 ± 0.02	0.64 ± 0.01	0.47 ± 0.01	0.51 ± 0.05	0.57 ± 0.02	0.63 ± 0.01	0.47 ± 0.01	0.44 ± 0.04	0.48 ± 0.06

3 m=3 months; 6 m=6 months; ST=Shear Thinning Index; Thix=Thixotropy Index

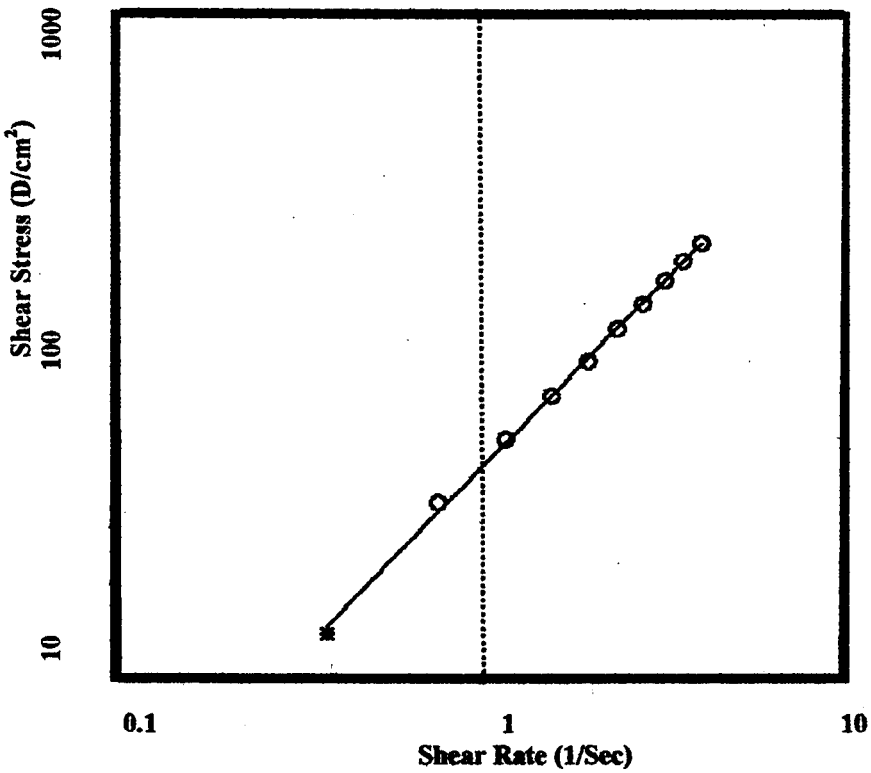


Figure 4.17. Rheogram of Freshly Prepared Base

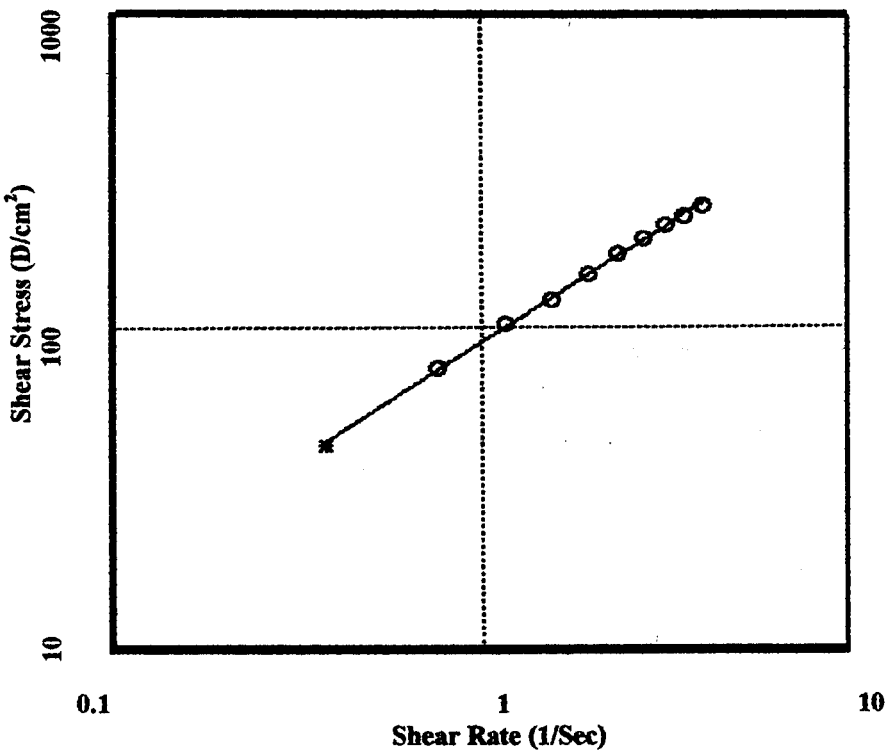


Figure 4.18. Rheogram of Freshly Prepared Formulation

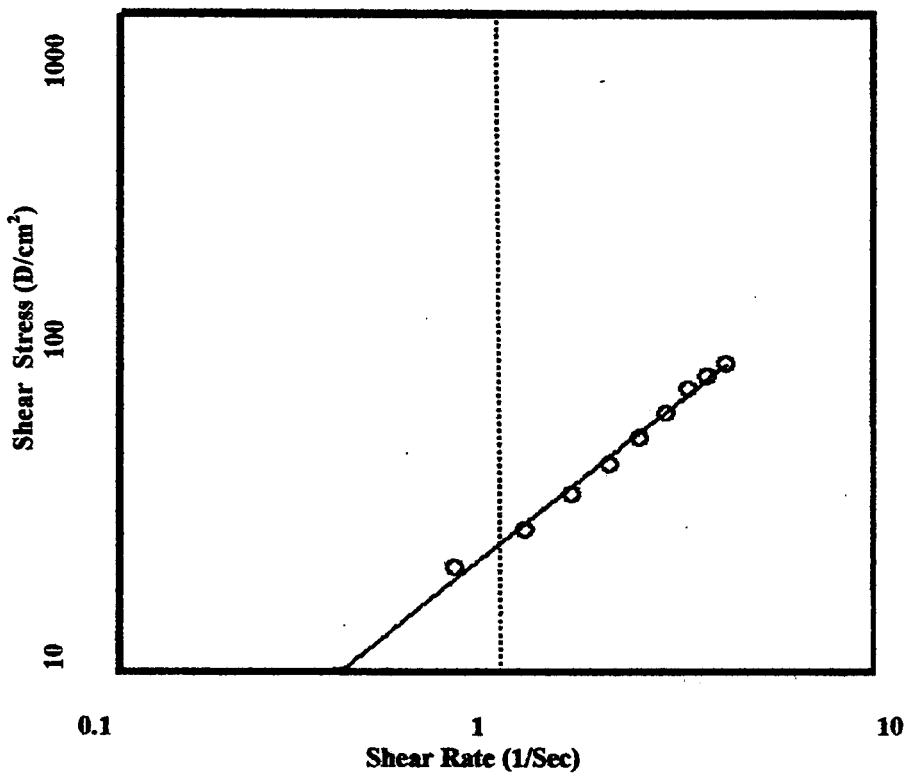


Figure 4.19. Rheogram of Base at 4^{0}C After 3 Months

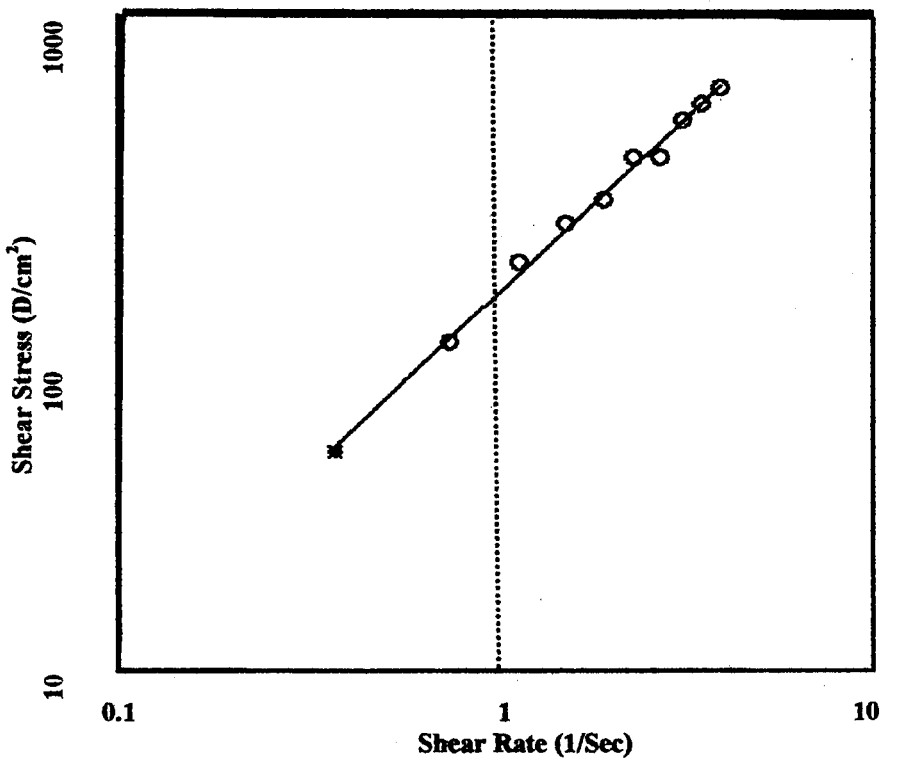


Figure 4.20. Rheogram of Formulation at 4^{0}C After 3 Months

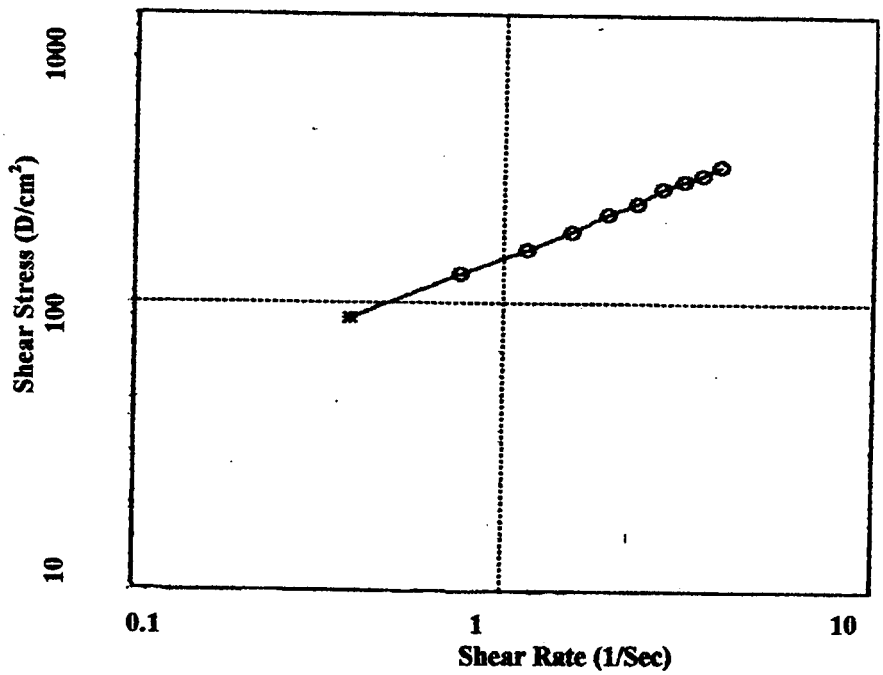


Figure 4.21. Rheogram of Base at 4°C After 6 Months

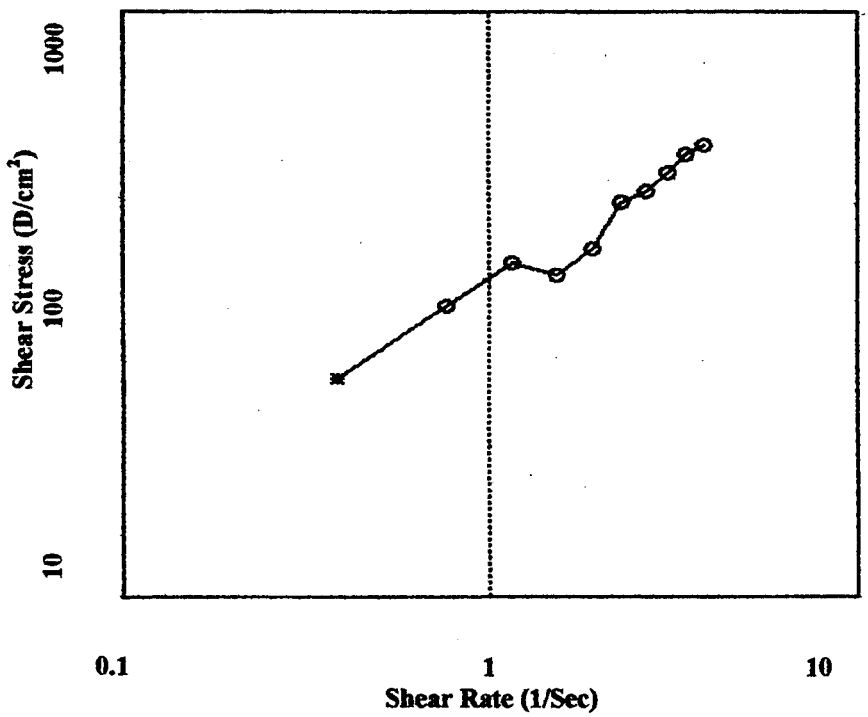


Figure 4.22. Rheogram of Formulation at 4°C After 6 Months

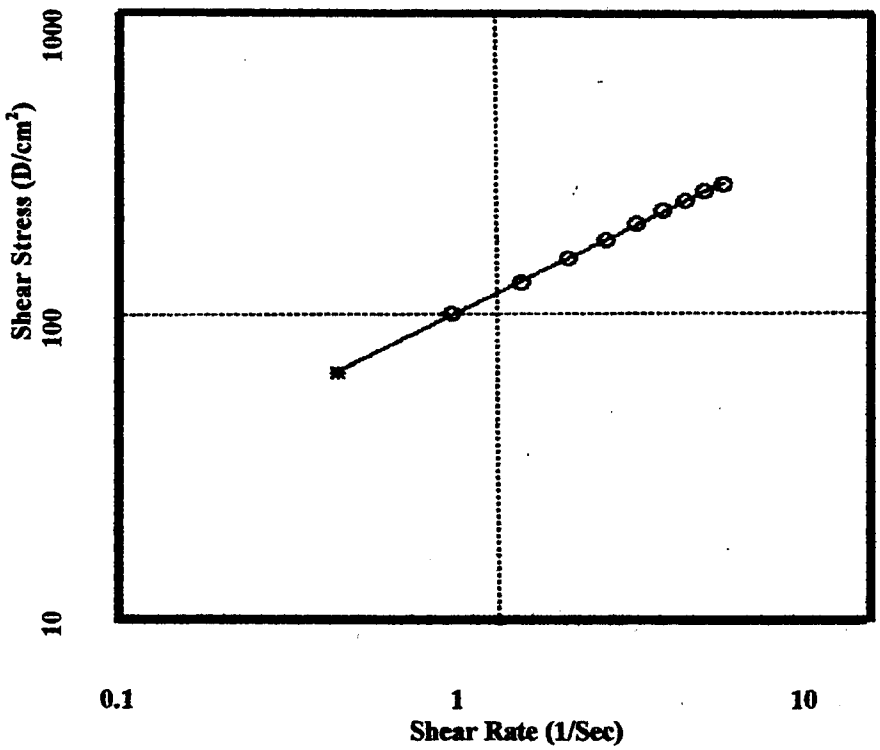


Figure 4.23. Rheogram of Base at 25^{0}C After 3 Months

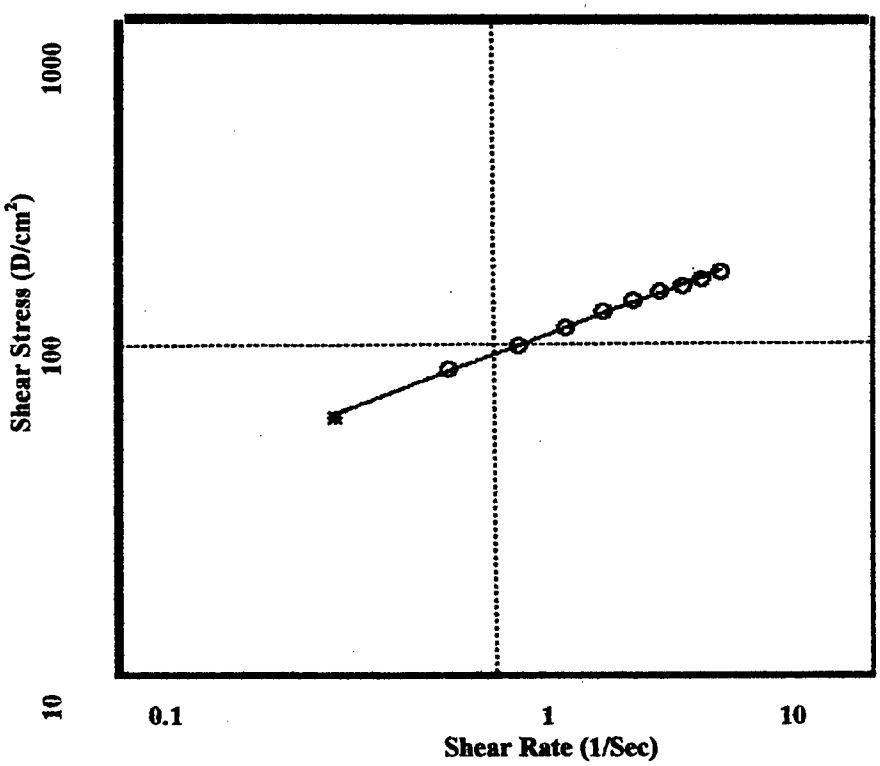


Figure 4.24. Rheogram of Formulation at 25^{0}C After 3 Months

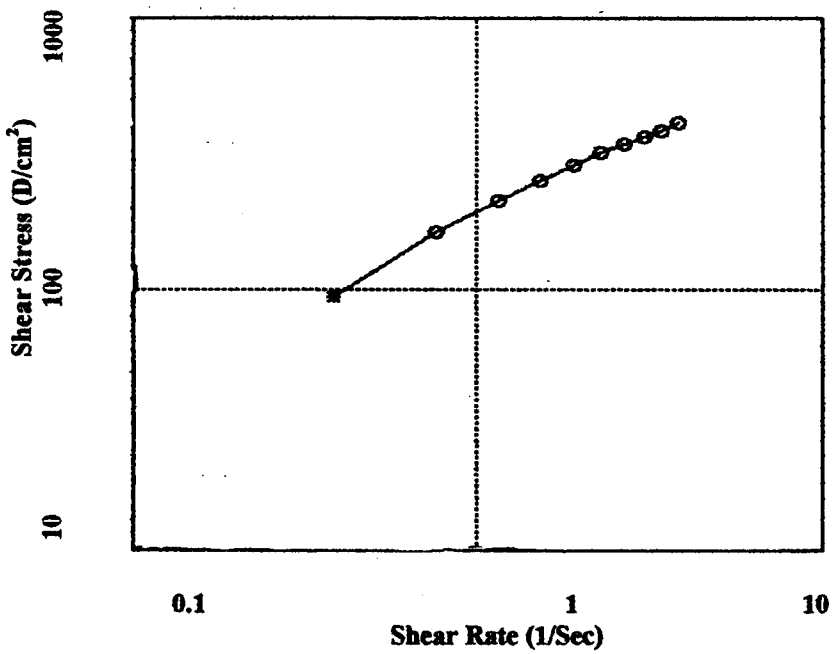


Figure 4.25. Rheogram of Base at 25^{0}C After 6 Months

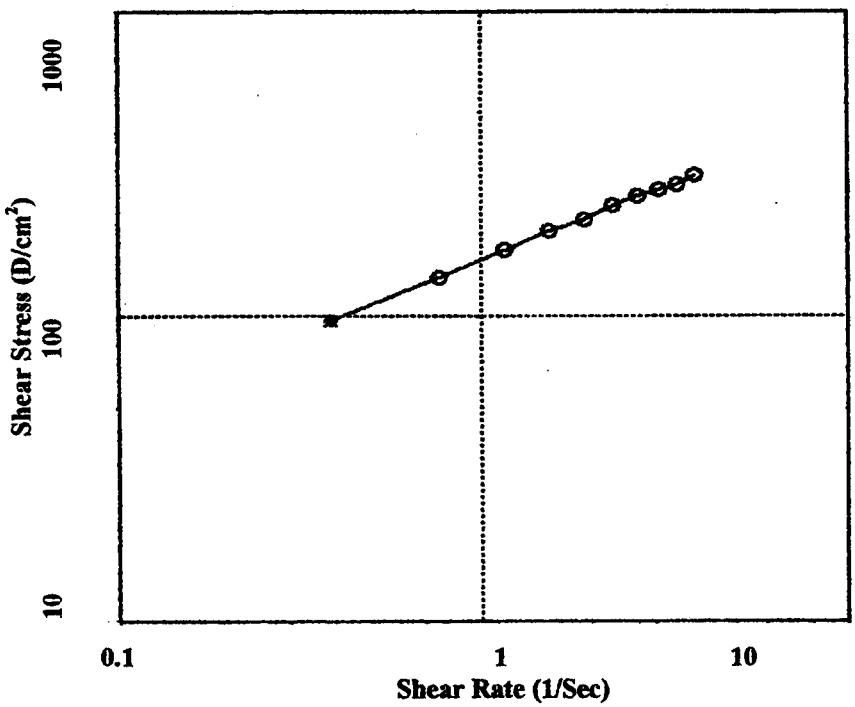


Figure 4.26. Rheogram of Formulation at 25^{0}C After 6 Months

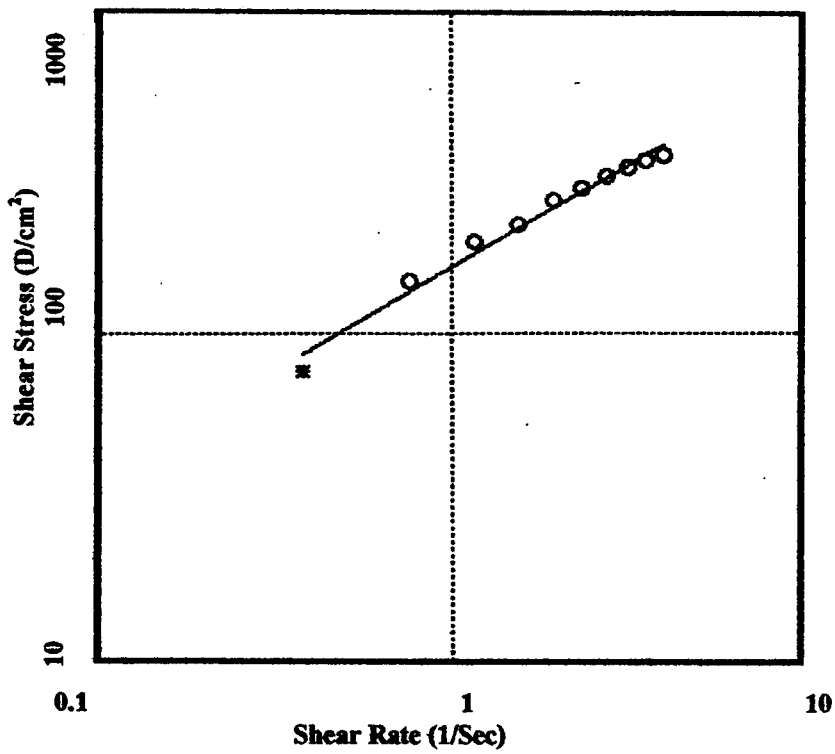


Figure 4.27. Rheogram of Base at 40^{0}C After 3 Months

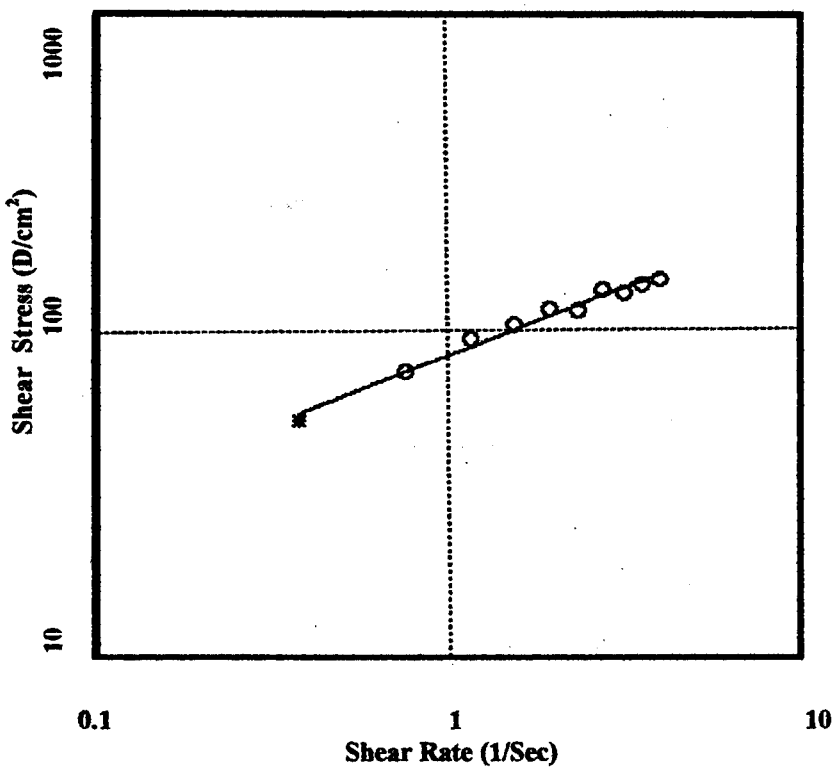


Figure 4.28. Rheogram of Formulation at 40^{0}C After 3 Months

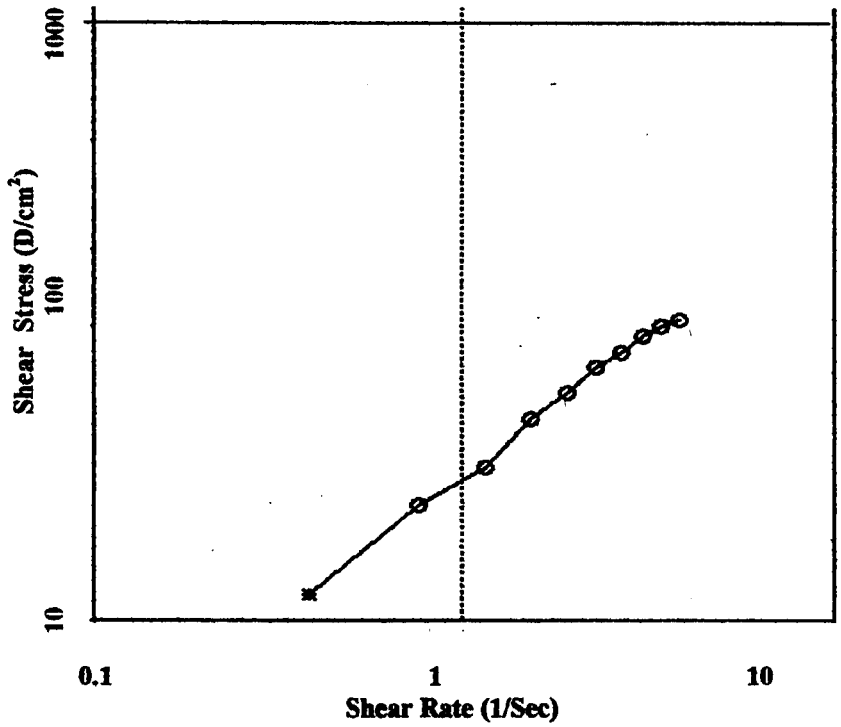


Figure 4.29. Rheogram of Base at 40^{0}C After 6 Months

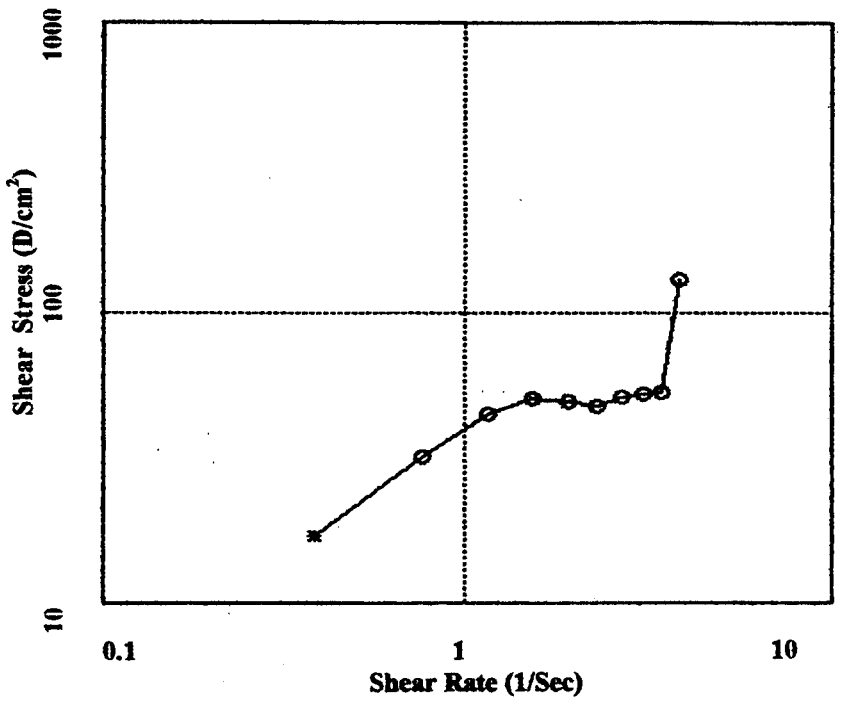


Figure 4.30. Rheogram of Formulation at 40^{0}C After 6 Months

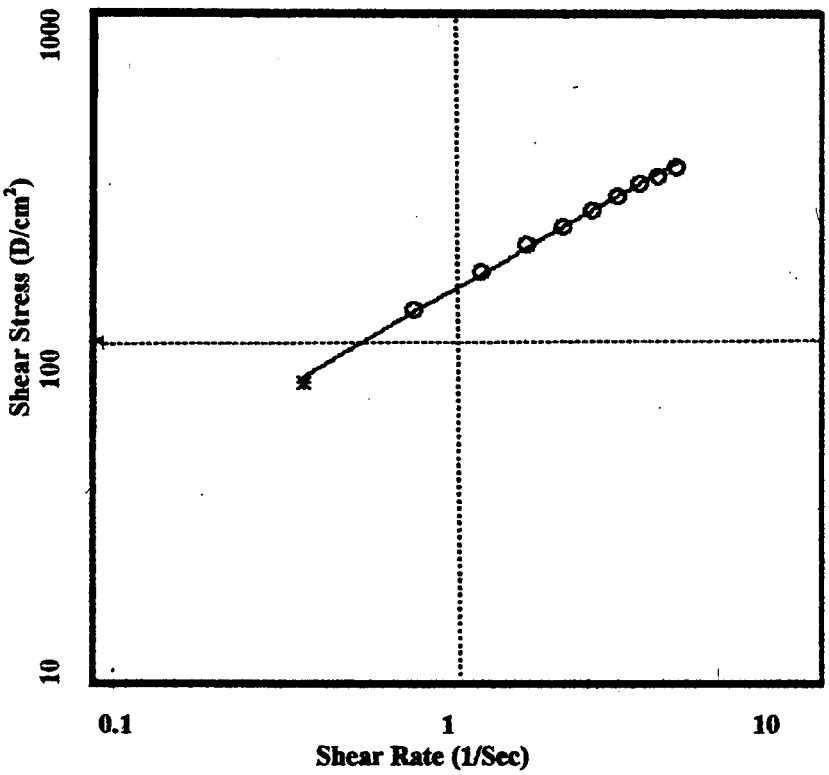


Figure 4.31. Rheogram of Base at 40°C & 60 % RH After 3 Months

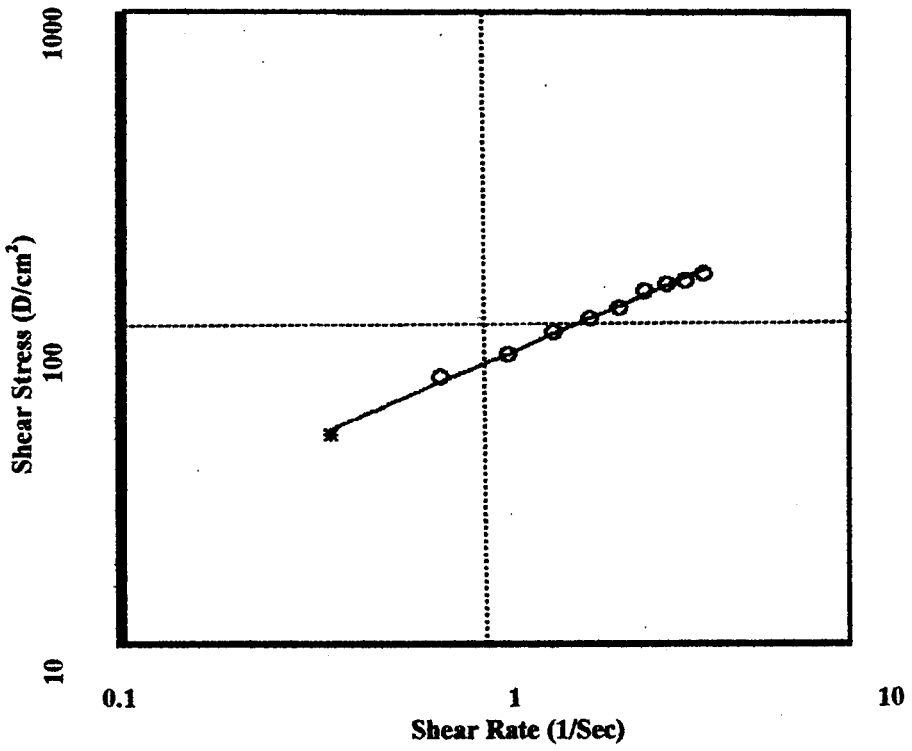


Figure 4.32. Rheogram of Formulation at 40°C & 60 % RH After 3 Months

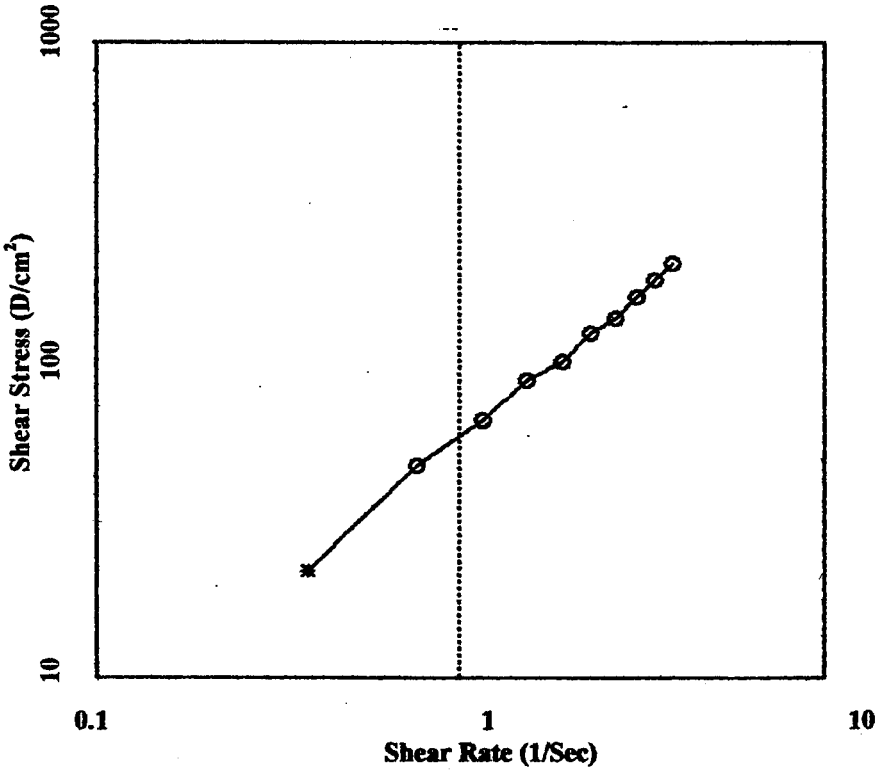


Figure 4.33. Rheogram of Base at 40^{0}C & 60 % RH After 6 Months

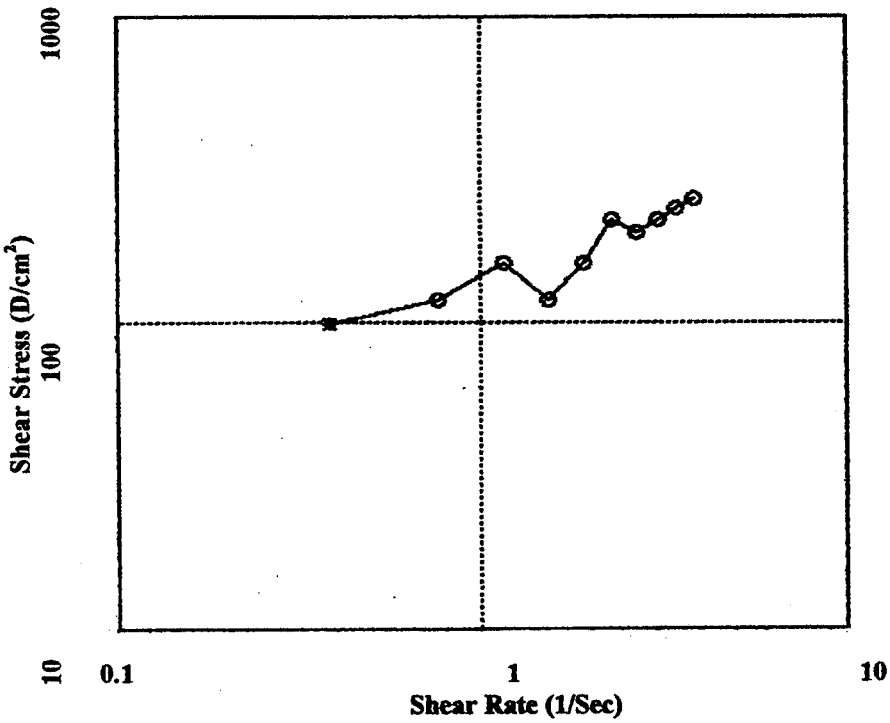


Figure 4.34. Rheogram of Formulation at 40^{0}C & 60 % RH After 6 Months

4.1.6. Dermatological Tests

4.1.6.1. Patch Test for Erythema and Melanin

Before the application of formulations to human volunteers, patch tests for erythema and melanin were performed and the values measured have been given in **Table 4.7.** Percentages of change in Melanin/Erythema have been shown in **Figure 4.35.**

Table 4.7. Percentages of Change in Melanin/Erythema After 24 Hours

	Base		Formulation	
Volunteers	**Melanin**	**Erythema**	**Melanin**	**Erythema**
Y.Y.	-0.51	0.14	0.40	2.20
A.B.	0.87	-0.42	0.48	-0.63
K.E.	1.57	-1.30	1.58	-1.84
H.T.	0.26	0.89	1.0	1.28
N.F.	-0.32	1.28	0.58	1.12
F.A.	-0.20	-0.92	1.05	0.75
M.A.	0.80	-1.11	-0.22	-0.06
I.A.	-0.34	1.83	-1.0	3.57
N.A.	-1.25	2.74	-1.55	3.65
H.Ö.	-0.52	1.08	-0.15	3.62
G.E.	-0.47	-0.60	2.14	2.30
Mean ± SE	**-0.01 ± 0.24**	**0.33 ± 0.40**	**0.39 ± 0.33**	**1.45 ± 0.55**

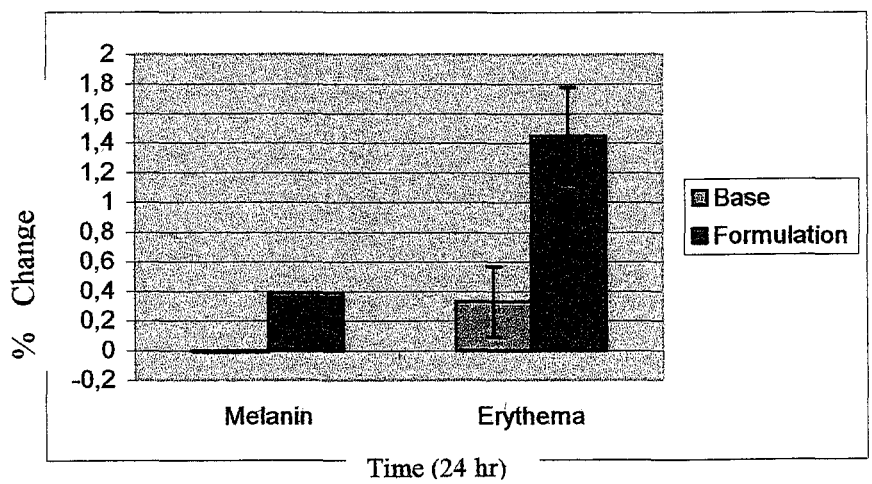


Figure 4.35. Percentage of Change in Melanin/Erythema After 24 Hours

4.1.6.2. Erythema and Melanin

The percentages of changes in the amounts of erythema and melanin following the applications of the formulations on the cheeks of human volunteers have been demonstrated in **Tables 4.8. and 4.9.** and **Figure 4.36. and 4.37.**

Table 4.8. Percentages of Change in Melanin/Erythema After Application of Base

Volunteer	Melanin				Erythema			
	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week
Y.Y.	0.76	-0.30	0.30	1.14	-2.28	-1.55	-1.14	0.05
A.B.	1.43	1.69	2.51	1.84	1.06	1.27	1.92	2.88
K.E.	1.59	0.06	0.16	0.92	-1.48	2.27	-0.05	1.01
H.T.	0.97	1.62	1.68	2.79	1.84	0.87	-0.08	-1.67
N.F.	0.36	-0.76	0.50	0.18	-0.62	-0.33	-0.98	-0.98
F.A.	0.50	0.10	1.14	1.24	-1.18	-0.66	-1.34	-0.66
M.A.	1.19	1.06	0.50	0.10	-0.72	-1.32	-2.30	-0.60
I.A.	-0.75	-1.18	0.43	0.89	1.57	3.96	4.04	4.01
N.A.	1.62	-0.24	0.10	-0.14	1.78	1.46	2.40	1.83
H.Ö.	0.74	-1.33	-0.77	-1.20	-3.79	-2.49	-1.77	-1.69
G.E.	0.51	0.31	1.23	1.02	-0.40	-3.85	-0.13	-0.48
Mean ± SE	**0.81 ± 0.21**	**0.10 ± 0.31**	**0.71 ± 0.27**	**0.80 ± 0.32**	**-0.38 ± 0.55**	**-0.03 ± 0.68**	**0.05 ± 0.59**	**0.34 ± 0.57**

Table 4.9. Percentages of Change in Melanin/Erythema After Application of Formulation

Volunteer	Melanin				Erythema			
	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week
Y.Y.	1.87	1.12	1.47	2.59	-2.37	-2.15	-0.17	-1.60
A.B.	0.10	1.13	1.15	0.85	-1.29	2.18	1.15	3.00
K.E.	1.68	1.17	0.92	1.33	-2.38	0.78	-0.05	-0.67
H.T.	0.56	0.56	1.31	1.21	1.82	2.82	0.69	0.43
N.F.	-0.94	-1.91	-0.76	-0.82	0.60	0.12	-1.55	-0.65
F.A.	-2.50	-2.50	-1.93	-1.35	1.32	0.53	-0.05	0.33
M.A.	0.30	0.60	0.36	0.96	-1.80	-1.40	-2.14	-0.81
I.A.	-2.08	-1.55	-1.94	-0.54	0.83	2.94	2.89	0.59
N.A.	-0.45	-1.15	-1.01	-1.01	-1.44	0.0	1.40	-1.25
H.Ö.	-0.42	-1.86	-1.71	-1.17	-2.07	-1.06	-0.78	0.0
G.E.	1.74	1.74	1.54	2.71	-2.86	-4.17	-3.70	-4.75
Mean ± SE	**-0.01 ± 0.44**	**0.24 ± 0.47**	**-0.06 ± 0.43**	**0.43 ± 0.45**	**-0.88 ± 0.51**	**0.05 ± 0.66**	**-0.21 ± 0.55**	**-0.54 ± 0.59**

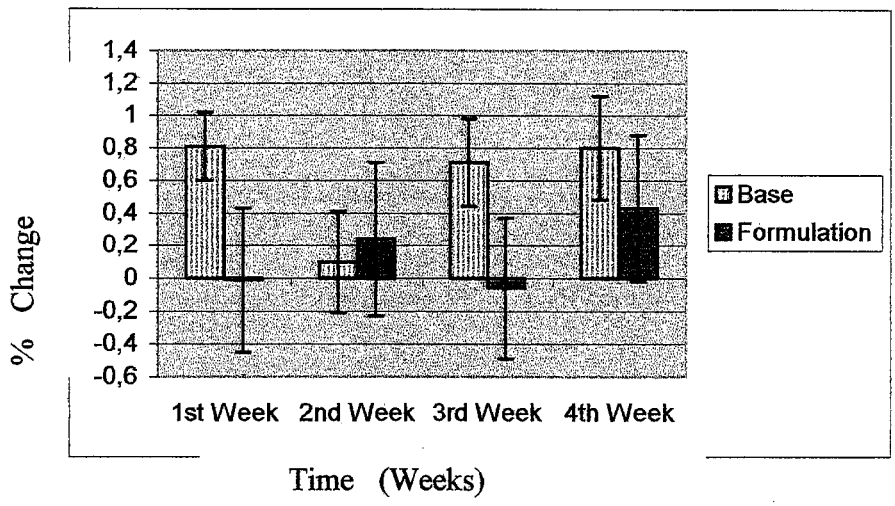


Figure 4.36. Percentage of Change in Melanin After Application of Preparations

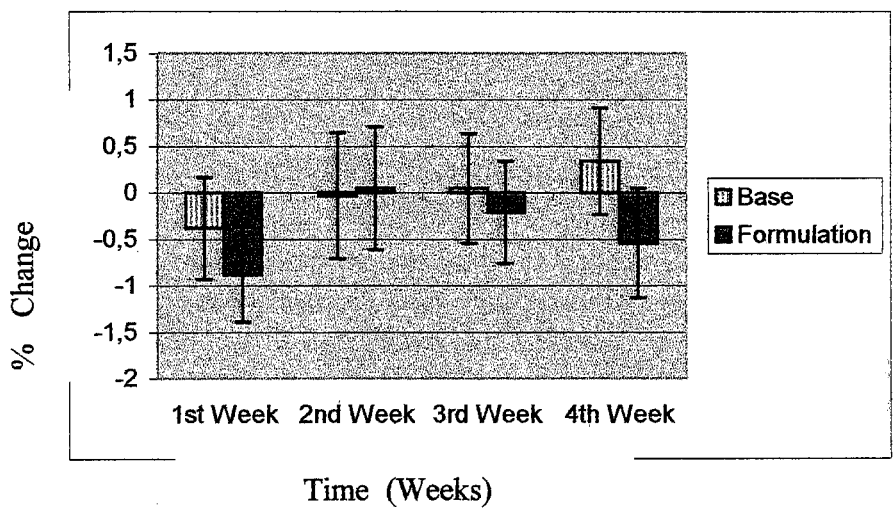


Figure 4.37. Percentages of Change in Erythema After Application of Preparations

4.1.6.3. Skin Moisture

The percentages of changes in the measured skin moisture values before and after applications of formulations have been given in **Table 4.10.** and **Figure 4.38.**

Table 4.10. Percentages of Change in Values of Skin Moisture

Volunteer	Base				Formulation			
	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week
Y.Y.	-14.47	-24.48	-4.82	-5.82	-13.90	-3.87	-7.55	-3.87
A.B.	-2.06	19.36	18.83	11.02	-2.47	17.99	16.55	4.42
K.E.	2.62	13.10	9.96	1.42	6.41	9.50	12.71	8.62
H.T.	-32.73	-20.58	-2.88	7.46	-29.40	-5.32	-20.72	8.80
N.F.	6.29	23.83	7.01	31.64	16.82	46.50	20.91	50.58
F.A.	13.42	27.96	13.54	15.32	17.72	33.29	21.02	38.99
M.A.	-23.39	-4.24	3.27	10.20	-42.41	-16.09	4.30	8.59
I.A.	-27.06	-9.83	9.08	-4.40	-3.74	-2.76	8.56	-4.63
N.A.	-25.37	-13.43	20.40	4.97	-16.68	-19.77	14.21	11.12
H.Ö.	-14.14	2.64	21.63	22.63	5.17	14.93	35.02	32.03
G.E.	-24.79	-13.40	5.91	14.03	-24.18	-14.53	4.88	23.04
Mean ± SE	**-12.88 ± 4.69**	**0.09 ± 5.55**	**9.27 ± 2.67**	**9.86 ± 3.35**	**-7.87 ± 5.81**	**5.44 ± 6.38**	**9.99 ± 4.52**	**16.16 ± 5.36**

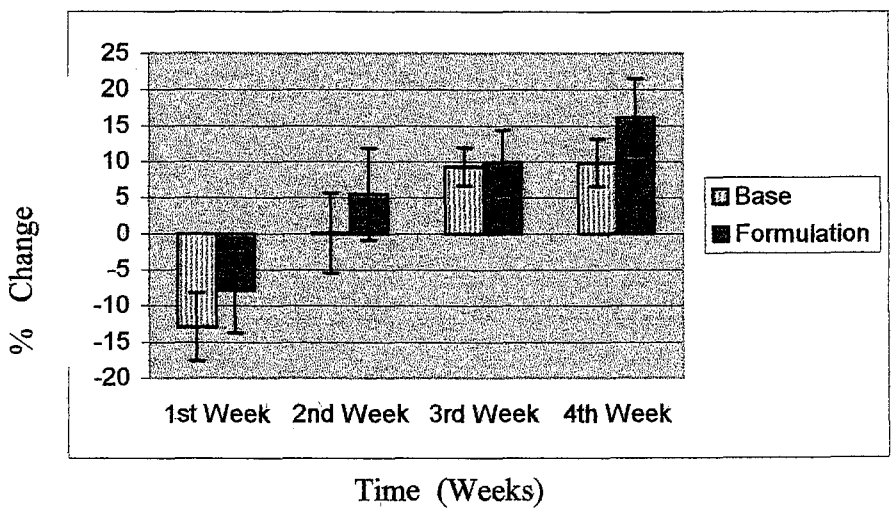


Figure 4.38. Percentages of Change in Values of Skin Moisture

4.1.6.4. Skin Sebum

The percentages of changes in the measured skin sebum values before and after applications of formulations have been given in **Table 4.11.** and **Figure 4.39.**

Table 4.11. Percentages of Change in Values of Skin Sebum

Volunteer	Base				Formulation			
	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week
Y.Y.	156.25	85.42	121.86	129.17	115.91	69.32	102.27	89.77
A.B.	6100.00	1100.00	800.0	7700.00	1060.00	0.00	0.00	1630.00
K.E.	1333.30	1308.30	583.33	633.33	525.00	975.00	300.00	875.00
H.T.	-31.19	3.67	-4.13	-66.06	-47.44	-16.03	-26.92	-25.64
N.F.	47.50	-3.75	-58.75	26.25	82.86	-20.00	5.71	102.86
F.A.	-2.97	-31.68	-19.80	-0.50	33.71	-33.15	-18.54	12.36
M.A.	-9.85	-32.96	-32.58	-11.36	9.67	-21.77	-20.57	-22.58
I.A.	94.37	42.96	14.09	18.31	51.25	0.00	-0.63	15.63
N.A.	-89.16	-59.04	-57.83	-63.25	-94.83	-75.86	-59.48	-32.76
H.Ö.	-58.90	36.30	-24.66	19.18	-70.51	33.33	-54.49	-33.97
G.E.	-84.16	-87.13	-96.54	-80.69	-68.42	-64.04	-93.86	-60.53
Mean ± SE	**677.70 ± 555.30**	**214.74 ± 148.70**	**111.37 ± 89.20**	**754.94 ± 696.40**	**145.20 ± 104.88**	**76.98 ± 90.53**	**13.35 ± 34.13**	**231.83 ± 161.00**

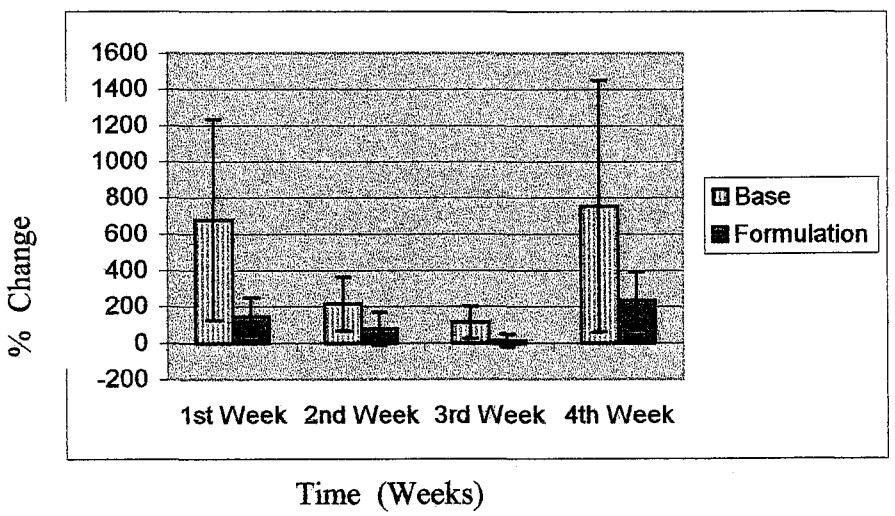


Figure 4.39. Percentages of Change in Values of Skin Sebum

4.1.6.5. pH of Skin

The percentages of changes in the skin pH values before and after applications of formulations have been given in **Table 4.12.** and **Figure 4.40.**

Table 4.12. Percentages of Change in Skin pH values

Volunteers	Base				Formulation			
	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week
Y.Y.	0.00	8.77	-7.01	-1.75	-3.50	3.50	-3.50	-3.50
A.B.	18.96	-1.72	-3.44	-5.17	17.85	3.57	3.57	0.00
K.E.	10.90	1.81	9.09	14.54	7.14	-1.78	7.14	10.71
H.T.	5.55	9.25	0.00	-5.55	5.55	9.25	-1.85	1.85
N.F.	-17.91	-19.40	-10.44	-22.38	-16.66	-16.66	-13.63	-19.69
F.A.	1.72	3.44	-1.72	-12.06	7.14	1.78	5.35	-7.14
M.A.	12.24	4.08	-2.04	-2.04	14.28	10.20	2.04	0.00
L.A.	11.53	3.84	3.84	-1.92	15.38	5.76	5.76	1.92
N.A.	6.89	-3.44	-15.51	-6.89	14.03	3.50	-10.52	0.00
H.Ö.	18.96	1.72	10.34	-1.72	13.55	0.00	8.47	0.00
G.E.	4.91	0.00	-4.91	-4.91	6.77	10.16	3.38	0.00
Mean ± SE	**6.71 ± 3.09**	**-0.76 ± 2.37**	**1.98 ± 2.34**	**4.53 ± 2.65**	**-7.41 ± 3.02**	**-2.66 ± 2.27**	**-0.57 ± 2.18**	**1.44 ± 2.23**

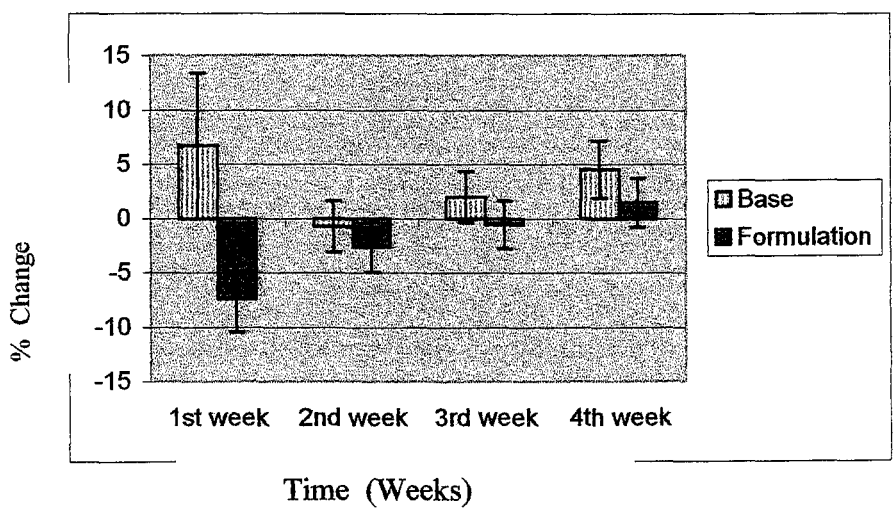


Figure 4.40. Percentages of Change in Skin pH Values

4.1.6.6. Skin Elasticity

The percentages of changes in the skin elasticity values before and after applications of formulations have been given in **Table 4.13.** and **Figure 4.41.**

Table 4.13. Percentages of Change in Values of Net Elasticity of Skin

Volunteers	Base				Formulation			
	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week
Y.Y.	-23.66	-18.20	12.04	-18.34	-3.50	-3.50	3.50	3.50
A.B.	-20.00	60.00	20.00	40.00	17.85	-3.57	-3.57	0.00
K.E.	13.43	-9.01	-20.74	19.04	7.14	1.78	-7.14	-10.71
H.T.	-22.17	-24.15	-19.95	-29.98	5.55	-9.25	1.85	-1.85
N.F.	116.5	72.29	88.96	71.64	-16.66	16.66	13.63	19.69
F.A.	-10.71	12.14	11.85	-7.71	7.14	-1.78	-5.35	7.14
M.A.	5.75	-1.07	27.33	13.84	14.28	-10.20	-2.04	0.00
I.A.	-6.29	4.94	3.44	-11.54	15.38	-5.76	-5.76	-1.92
N.A.	-31.36	9.60	20.00	4.00	14.03	-3.5	10.52	0.00
H.Ö.	-25.03	0.29	0.00	-2.72	13.55	0.00	-8.47	0.00
G.E.	-39.97	-21.96	-18.96	-6.96	6.77	-10.16	-3.38	0.00
Mean ± SE	**-6.71 ± 11.97**	**7.72 ± 9.48**	**11.27 ± 9.31**	**6.48 ± 8.69**	**7.14 ± 3.02**	**-2.66 ± 2.27**	**-0.57 ± 2.18**	**-1.44 ± 2.23**

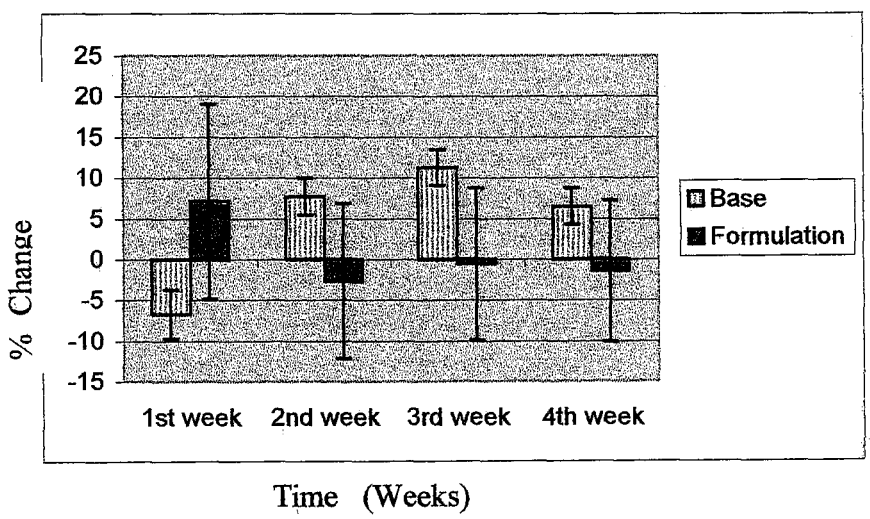


Figure 4.41. Percentages of Change in Values of Net Elasticity of Skin

4.1.6.7. Panel Test

Sensory evaluation of the two creams by the volunteers has been presented in **Table 4.14.** and **Figure 4.42.**

Table 4.14. Average Values ± SE for Panel Test

	Average point for Base	Average points for Formulation
Ease of Application	4.36 ± 0.20	4.46 ± 0.21
Spreadability	4.27 ± 0.20	4.36 ± 0.20
Sense just after application	3.64 ± 0.20	3.91 ± 0.25
Sense in long term	4.27 ± 0.20	4.18 ± 0.23
Irritation	0.0 ± 0.0	0.0 ± 0.0
Shine on skin	3.82 ± 0.23	3.73 ± 0.31
Sense of softness	4.82 ± 0.12	4.56 ± 0.28

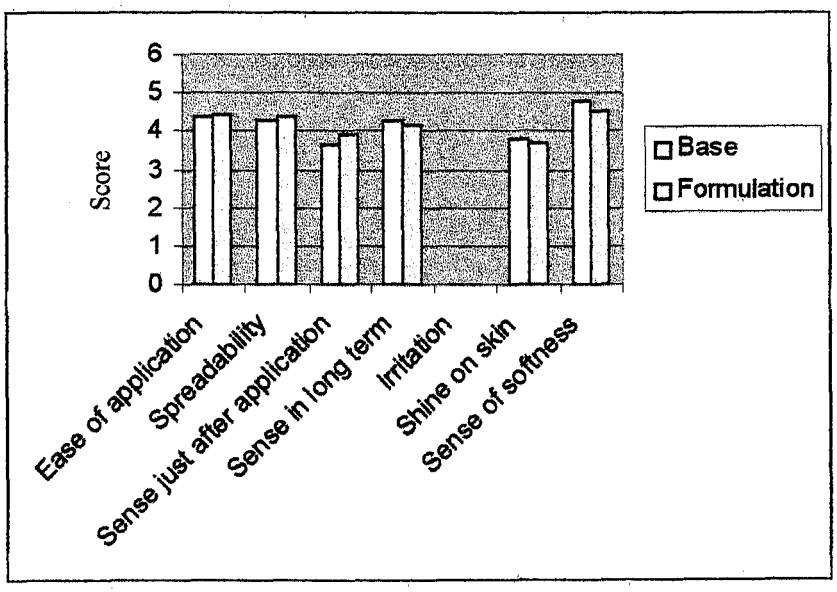


Figure 4.42. Average Values for Panel Test

4.1.6.8. Statistical Evaluation

Results of statistical evaluation has been shown in **Tables 4.15. - 4.20.**

Table 4.15. Statistical Evaluation of Values of Erythema After Application of Formulation

Weeks	Mean±SE	0 Week	1st Week	2nd Week	3rd Week	4 th Week
0 Week	615.33± 7.02		p>0.05	p>0.05	p>0.05	p>0.05
1st Week	-0.88± 0.51	p>0.05		p>0.05	p>0.05	p>0.05
2nd Week	0.05± 0.66	p>0.05	p>0.05		p>0.05	p>0.05
3rd Week	-0.21± 0.55	p>0.05	p>0.05	p>0.05		p>0.05
4th Week	-0.54± 0.59	p>0.05	p>0.05	p>0.05	p>0.05	

p>0.05=No Variance; p<0.05=Significant Variance; SE=Standard Error

Table 4.16. Statistical Evaluation of Values of Melanin After Application of Formulation

Weeks	Mean±SE	0 Week	1st Week	2nd Week	3rd Week	4 th Week
0 Week	508.66± 7.23		p>0.05	p>0.05	p>0.05	p>0.05
1st Week	-0.01± 0.44	p>0.05		p>0.05	p>0.05	p>0.05
2nd Week	0.24± 0.47	p>0.05	p>0.05		p>0.05	p>0.05
3rd Week	-0.06± 0.43	p>0.05	p>0.05	p>0.05		p>0.05
4th Week	0.43± 0.45	p>0.05	p>0.05	p>0.05	p>0.05	

p>0.05=No Variance; p<0.05=Significant Variance; SE=Standard Error

Table 4.17. Statistical Evaluation of Values of Skin Moisture After Application of Formulation

Weeks	Mean±SE	0 Week	1st Week	2nd Week	3rd Week	4 th Week
0 Week	93.7± 2.98		p>0.05	p>0.05	p>0.05	p<0.05
1st Week	-7.87± 5.81	p>0.05		p<0.05	p>0.05	p<0.05
2nd Week	5.44± 6.38	p>0.05	p>0.05		p>0.05	p>0.05
3rd Week	9.99± 4.52	p>0.05	p<0.05	p>0.05		p>0.05
4th Week	16.16± 5.36	p<0.05	p<0.05	p>0.05	p>0.05	

p>0.05=No Variance; p<0.05=Significant Variance; SE=Standard Error

Table 4.18. Statistical Evaluation of Values of Skin Sebum After Application of Formulation

Weeks	Mean±SE	0 Week	1st Week	2nd Week	3rd Week	4 th Week
0 Week	30.98± 11.97		p>0.05	p>0.05	p>0.05	p>0.05
1st Week	145.02± 104.88	p>0.05		p>0.05	p>0.05	p>0.05
2nd Week	76.98± 90.53	p>0.05	p>0.05		p>0.05	p>0.05
3rd Week	13.35± 34.13	p>0.05	p>0.05	p>0.05		p>0.05
4th Week	231.83± 161.00	p>0.05	p>0.05	p>0.05	p>0.05	

p>0.05=No Variance; p<0.05=Significant Variance; SE=Standard Error

Table 4.19. Statistical Evaluation of Values of Skin pH After Application of Formulation

Weeks	Mean±SE	0 Week	1st Week	2nd Week	3rd Week	4 th Week
0 Week	-5.65± 0.13		p<0.05	p>0.05	p>0.05	p>0.05
1st Week	-7.41± 3.02	p<0.05		p>0.05	p>0.05	p<0.05
2nd Week	-2.66± 2.27	p>0.05	p>0.05		p>0.05	p>0.05
3rd Week	-0.57± 2.18	p>0.05	p>0.05	p>0.05		p>0.05
4th Week	1.44± 2.23	p<0.05	p>0.05	p>0.05	p>0.05	

p>0.05=No Variance; p<0.05=Significant Variance; SE=Standard Error

Table 4.20. Statistical Evaluation of Values of Net Elasticity After Application of Formulation

Weeks	Mean±SE	0 Week	1st Week	2nd Week	3rd Week	4 th Week
0 Week	0.65± 0.04		p>0.05	p>0.05	p>0.05	p>0.05
1st Week	7.14± 3.02	p>0.05		p>0.05	p>0.05	p>0.05
2nd Week	-2.66± 2.27	p>0.05	p>0.05		p>0.05	p>0.05
3rd Week	-0.57± 2.18	p>0.05	p>0.05	p>0.05		p>0.05
4th Week	-1.44± 2.23	p>0.05	p>0.05	p>0.05	p>0.05	

p>0.05=No Variance; p<0.05=Significant Variance; SE=Standard Error

4.2. DISCUSSION

4.2.1. Basic Properties of Macadamia Nut Oil

4.2.1. Rheological Analysis

Rheology of dispersed systems is among their most important physical properties which influences not only the physical stability of the system, but also the performance, quality and utility of the product (107).

It is known that greater the viscosity of the oil, the greater is the viscosity of the emulsion. It was found that there is a high level of correlation between the viscosity of cosmetic oils and experienced greasiness (229).

Continuous phase plays a dominant role in the rheology of an emulsion even though each component contributes to the emulsion's rheology. Viscosity of the emulsion is directly proportional to the viscosity of the continuous phase. The dispersed phase affects the rheology of the emulsion by its volume concentration, globule size, viscosity and chemical constitution (228).

Macadamia nut oil showed Newtonian flow as can be seen in **Figure 4.1.** Natural cosmetic oils are usually Newtonian liquids with relatively low viscosities (225).

Newtonian liquids show an ideal flow where shear stress is proportional directly to the shear rate,

$$\sigma = \eta\gamma$$

where σ is the shear stress, γ is the shear rate and η is the absolute viscosity (229). Viscosity is independent of the shear rate and was found to be 40.42 mPas.s for the macadamia nut oil whereas the viscosities of other natural oils like almond and avocado oils were 54.6 mPas.s and 98.4 mPas.s, respectively (229). This shows that the viscosity of macadamia nut oil is lower when compared to other natural oils.

4.2.1.2. Surface Tension

Surface tension (or surface energy) is defined as the work required to increase the area of a surface isothermally and reversibly by unit amount (230). Capillary rise method is the most accurate method for determining surface tensions, when performed properly. This method of measurement allows to follow time effects since no surface disturbance is involved (231).

The interaction between two liquid phases forms the basis of emulsion science. The tendency for one liquid to spread over another, which is oil and water in our case, is designated by a spreading coefficient, S (231). The spreading coefficient is

$$S = W^{adh} - W^{coh}$$

where W^{adh}, work of adhesion is the work required to separate the unit area of the two liquids by pulling them apart; and W^{coh}, work of cohesion, is the work required to separate the unit area of two liquid surfaces from itself. For liquid 1 and 2

$$W_{12}^{adh} = \gamma_1 + \gamma_2 - \gamma_{12}$$

Where γ_1 is the surface tension of liquid 1, γ_2 is the surface tension of liquid 2 and γ_{12} is the interfacial tension between 1 and 2

$$W_1^{coh} = 2\gamma_1$$

$$W_2^{coh} = 2\gamma_2$$

Spreading coefficient indicates how readily oil can spread over water. Calculations of this parameter then leads to the formulation of stable emulsion systems.

Surface tension of macadamia nut oil was found to be 33.03 mN/m **(Table 4.1.)** whereas the surface tensions of other natural oils like almond and avocado oils were found to be 36.3 mN/m and 34.6 mN/m respectively (229). It means that there is no big difference between the surface tensions of macadamia nut oil and other natural oils.

4.2.1.3. Spreadability

Since spreadability depends on external conditions such as temperature and atmospheric humidity, *in vitro* spreadability values were determined in a conditioned room of 52% relative humidity and 23^0C temperature. The spreadability of the tested oil was assigned to a group according to the diameter found (228).

I	<10 mm	Low spreadability
II	10-13 mm	Moderate spreadability
III	13-16 mm	Good spreadability
IV	16-19 mm	Very good spreadability
V	>19 mm	Super spreadability

Diameter of the spreading area was found to be 8.2 mm ± 0.02 mm (SE). This value shows that macadamia nut oil belongs to group 1 of low spreadability.

The spreadability of an oil is in correlation with the subjective evaluation of penetration capability into *Stratum corneum* and the time course of the greasy feeling (228).

4.2.1.4. Pour Point and Cloud Point

Pour point specifies the temperature at which the oil solidifies and is defined as the temperature on cooling, with 3^0C added thereto, at which the substance is no longer able to flow (DIN ISO 3016).

Cloud point of an oil is defined as the temperature at which the substance becomes turbid on cooling (EN 23015) (228).

Direct relationship between freeze stability of water-in-oil emulsions and pour point of the oils were reported (229). Therefore it was concluded that if the pour point of the oil is low, solidification point of the emulsion will be low and thus the freeze stability of the emulsion will be better.

Pour point of macadamia nut oil was found to be -1.83^0C $\pm 0.17^0$C (SE) and cloud point was 0.17^0C $\pm 0.17^0$C (SE). The pour and cloud points of almond oil were reported to be -20^0C and -18.5^0C; -15.5^0C and -13.5^0C for avocado oil (229). This indicates that the pour and cloud points of macadamia nut oil are not very low leading to low freeze stability as compared to other natural oils like almond and avocado oils.

4.2.1.5. Polarity

The polarity of the oil phase has a great influence on the formulation and properties of the cosmetic emulsions. Polarity of the oil phase is considered as an essential factor for the stability of water-in-oil emulsions (228).

Polarity of macadamia nut oil was found to be 525.50 nm±0.29 nm (SE). This is considered as highly polar. Polar oils may enhance solubility of oil soluble cosmetic ingredients. Heat stability of emulsions prepared with strongly polar and non-polar oils were found to give emulsions with poor stability which is generally experienced with natural oils (228).

4.2.2. Globule Size

Globule size remains the same as an indication of stability of multiple emulsions. The increase or decrease in the globule size indicates the process of instability (94). The multiple droplets may coalesce with the other oil drops; or the internal aqueous droplets may be expelled individually; or more than one drop may be expelled; or the internal globules may coalesce before being expelled out resulting in the shrinkage of internal droplets; or the water may pass from the external phase to the internal aqueous phase resulting in the swelling of internal droplets and then complete rupture of droplets.

Globule sizes can be determined by light microscope (110, 112) or by electron microscope (126,127). Light scattering and diffraction methods are also used for the determination of globule sizes (112). Coulter counting technique is also a practicle method for the determination of particle sizes (109).

In our study, an equipment utilizing light scattering and diffraction methods has been used. The average globule size of the freshly prepared base was 12.29 μm which is in the range of average globule size of multiple emulsions (108). The average globule size of base stored at different conditions increased with time which indicated coalescence of globules as defined in literature which ultimately shows the instability of multiple emulsion (94).

In the active formulation, the average globule size has decreased with time upto two months. After two months, there was again increase in the average globule size. This indicates that the mechanism of instability in the first two months is the shrinkage of globules, possibly due to the expulsion of internal

aqueous droplets to the external water phase. However, later on after two months, the mechanism of instability is coalescence of oil droplets as the globule size was increased. The most prominent increase in globule size was seen in sample placed at 4^0C after three months.

4.2.3. pH

pH values of the facial skin ranges between 5 and 6, and 5.5 is considered to be the average pH of the skin. Therefore, the formulations which are applied to the face are planned to have a pH in the range of 5-6. pH values of multiple emulsions were adjusted by triethanolamine to bring them to higher values and citric acid was added to bring the pH value towards the acidic values (107). pH value of the formulation plays an important role for many enzymes and proteins to work efficiently. If the pH value of the formulation is not in the optimum range, proteins in the formulations can not produce the required activity. For example, α-hydroxy acids produce their activities best at pH 3. If their pH values are increased, their activities are reduced (66).

pH values of the freshly prepared base was 6.64 which is near to the pH range of the skin. pH value of the base at different conditions has decreased with time.

pH of the freshly prepared active formulation was found to be 2.66 Therefore, it was adjusted to 6.27 using triethanolamine. pH value of the formulation also decreased with time for samples stored at different conditions. The most prominent decrease in pH was seen in the sample kept at 4^0C.

The decrease in pH may be due to the diffusion of water from internal phase to the external phase as the pH of distilled water ranges from 5-7. pH of the distilled water used in the preparation of emulsions was 4.65. The other theory which can be presented for the decrease in the pH values of the emulsions is the production of highly acidic by-product from any of the ingredients of the oil since the pH of base which contained no active material also decreased with time.

4.2.4. Stability

Multiple emulsions of w/o/w may deteriorate by several possible mechanisms (94) which include swelling of internal drops due to the osmotic pressure leading to passage of water from external phase to internal phase. Other

mechanisms include rupture of the oil layer or coalescence of the oil globules or the coalescence of the internal water droplets. Presence of electrolytes plays an important role in the stability of multiple emulsions by balancing the osmotic pressure between the internal and external aqueous phases or by forming a rigid interfacial layer between oil and internal aqueous phase (107). The osmotic pressure gradient between the two aqueous phases may lead to a dynamic change in the volume of the internal aqueous phase due to the migration of water through the oil layer (117). Transfer of the drug to the external aqueous phase can occur either by total breakdown of the multiple droplets or by diffusion of the unionized drug through the oil layer (120).

Although diffusion is the most important mechanism of transport in the multiple emulsion systems, there is evidence that ionized materials may pass across the oil layer. Kita et al (124) have suggested that ionized drug along with water is mixed with micelles of hydrophilic and hydrophobic surfactants and cross the oil layer. Another possible transport mechanism is carrier mediated transport (125). The drug is bound with the carrier and this complex is permeable to the oil membrane. After passing through the oil membrane, the carrier is transported back. In this way the carrier effectively 'pumps' the permeating compound.

In our work, the base and the formulation were divided into four parts and each sample was placed at 4^0C in refrigerator, at 25^0C in oven, at 40^0C in another oven and at 40^0C & 60 % RH in a cabinet. These samples were analysed for six months at definite intervels. These samples were analysed with respect to change in color, liquifaction and phase separation.

4.2.4.1. Color

Regarding the base, there was no change in the color of the emulsion upto six months in any of the samples. The color was white throughout the period of analysis, i.e. six months. This shows that the base is stable at all different conditions.

Regarding the formulation, there was little change in the color of the sample kept at 4^0C upto 6 months. The change in color appeared after three months. The color of the formulation became yellowish-white which persisted upto six months of analysis period. The sample at 25^0C remained the same upto 3

months but there was a small change in color after three months as the sample turned yellowish-white. The color of the sample kept at 40^0C in the oven was changed within 15 days. Also, the color of the sample at 40^0C & 60 % RH was changed within 15 days. This change was from white color to yellowish-white. The change of color may be due to the presence of vitamin C in the external phase as some of the material in the internal aqueous phase may be lost into the external phase (107).

4.2.4.2. Liquifaction

Regarding the samples of base at different conditions, there was no liquifaction seen in any of the samples. The samples remained the same upto six months.

On the other hand, there was no change in the samples of formulation stored at 4^0C upto 30 days. After one month, the active emulsion showed liquifaction. The liquifaction has increased with time as noted after two months. Also, for the samples kept at 25^0C, liquifaction was seen after one month but there was no further increase in liquifaction upto six months. The samples at 40^0C also showed liquifaction in 15 days. The liquifaction has increased with time upto six months. The same pattern of liquifaction was seen in the samples kept at 40^0C & 60 % RH, i.e. increase in liquifaction with time.

This liquifaction which is a sign of instability, may be due to the passage of water from the internal phase to the external aqueous phase as has been described by many workers (117, 124).

4.2.4.3. Phase Separation

No complete phase separation was seen in any of the samples of the base stored at different conditions upto a period of six months. Similarly, no phase separation was visible in any of the samples of the active formulation kept at different conditions upto a period of six months. However, there was a tendency of phase separation in the samples of formulation at 4^0C after a period of three months. This tendency of phase separation is realised by the visuality of coalescence of globules. This indicates that emulsion of formulation is stable upto a period of six months at all conditions, regarding the phase separation.

4.2.5. Rheology

Rheological analyses are necessary to define and optimize stable multiple emulsions. These analyses allow the characterization of multiple emulsions, to follow changes in multiple emulsions induced by aging, shear and temperature and to predict their stability. Moreover, the rheological properties allow to describe and control the disruption mechanisms of the oily globules, which occur either by an osmotic swelling or simple shear flow (107).

In this study, a computerized cone-plate rheometer was used. All the rheological tests were performed at 25^{0}C. Increasing shear stresses were applied to the samples and the changes in viscosities were noted. These rheological tests were performed on freshly prepared base and on samples stored at different conditions after three and six months. Rheograms of shear stress versus shear rate were obtained. Shear thinning, thixotropy and flow indices were calculated for each rheogram. Then, different mathematical models were applied to the rheograms. Power Law was found to fit to all the rheograms and the confidences of fit were found to be in the range of 94.9-99.2 %.

The same tests were performed for the freshly prepared active formulation and for the samples kept at different conditions, after three and six months. Viscosities were found to decrease in parallel to the increase in shear stress. The viscosities were also found to decrease in samples kept at different conditions. Power Law was found to fit all the rheograms. Different parameters of viscosity, i.e. shear thinning, thixotropy and flow indices were calculated. Confidences of fit were found to be in between 96.2 and 99 %.

Shear thinning index increased in all the samples of both the formulations at different conditions with time. Thixotropy index decreased with time for base kept at different conditions while it increased with time for active formulation kept at different conditions. Flow index of the active formulation was most affected for the sample at 4^{0}C after 3 months.

4.2.6. Dermatological Tests

4.2.6.1. Skin Moisture

In the formulation of this study, vitamin C has been used which is known to increase collagen fibers in the dermis (9, 187, 192, 193, 222). With the increase

in collagen, the hydration conditions in the dermis are improved (212). In addition to this, vitamin C improves the barrier functions of the *Stratum corneum* thus improving the moisture in the skin (219). However, in our study, it was found that there was an increase in moisture values in the 4th week after the application of formulation as ANOVA test showed significant variation with respect to the basic values **(Table 4.17.)** but there was no significant difference in the moisture values when base was compared with active formulation as shown by t-test. This is in accordance with the reports already presented (212, 219). It is concluded that this increase in moisture values of formulation is due to vitamin C since Lipacide® does not act as moisturizer at this concentration (225).

4.2.6.2. Skin Sebum Content

Sebum production is measured using a special opalescent plastic film, which becomes transparent when it is in contact with sebum lipids (13). The device relies on a probe which presses a piece of special film on the skin for a measured length of time. The sebum is adsorbed on this film like ink on the blotting paper and the film becomes transparent. The probe is then placed into the device which radiates a light beam onto the film. A metal mirror behind the film reflects the beam back again through the film and then into an instrument called a photomultiplier, which measures the amount of light in the beam. The more sebum on the skin, the more transparent is the film and greater is the amount of light reflected.

The effects of formulations on the sebum contents of human cheeks were investigated in this study. It was found that the active formulation produced no significant effect on the secretion of the sebum with respect to the base in a period of 4 weeks application. Sebum was measured every week in all the individuals and with the help of ANOVA test it was found that there was no significant effect of formulation on the skin sebum values **(Table 4.18.)**; with paired sample t-test it was found that there was no significant variation between the active formulation and base regarding skin sebum content. This shows that neither vitamin C nor wheat proteins had any effect on the production of sebum using the multiple emulsion prepared.

4.2.6.3. Melanin

Melanocytes are present in the basal layer of the epidermis. These melanocytes manufacture a special pigment called melanin which is responsible for the color of the skin.

In this study, the effect of the active formulation on the production of melanin was examined. The amount of melanin was measured for 4 weeks in each individual and with the help of ANOVA test it was found that there was no significant effect of formulation on melanin **(Table 4.16.)**; and with the help of paired sample t-test it was found that there was no variation between the two creams. This showed that none of the two ingredients has any effect on melanin.

4.2.6.4. Erythema

For confirming the safety of cosmetics, the important point is that cosmetics must not cause any contact dermatitis when applied to the skin. The cause of contact dermatitis is not always due to cosmetic ingredients. Even if the safety of cosmetics is verified, it is known that environmental conditions such as temperature and humidity, misuse by the consumer, and the physical conditions may all cause contact dermatitis. Skin irritation is caused by the direct toxicity of chemicals on cells or blood vessels in the skin and is different from contact allergy which is caused by immune response. Vitamin C is used to act as anti-inflammatory (215, 221, 223).

In this study, patch tests were performed on the forearms of volunteers for 24 hours for both the formulation and the base. It was found that no irritation was produced by the two creams. In addition to this, irritation was constantly monitored every week for both the active formulation and the base for 4 weeks throughout the period of application. With the help of ANOVA test, it was found that neither active formulation nor base produced any irritation **(Table 4.15.)**; which is in accordance with reports already presented (215, 221, 223) and with the help of paired sample t-test it was evident that there was no significant variation in irritation with respect to base in any volunteers for a period of 4 weeks. This showed that the formulation prepared is safe and can be used without any side effects.

4.2.6.5. Skin pH Value

Sebum which is produced by sebaceous glands and is mixture of waxes and esters is slightly acidic. It has a pH of 4.2-5.6 (15).

pH values of the skins of volunteers were measured before and after application of formulations. It was found that the active formulation as well as the base did not produce any alteration on the pH values of human skin. Therefore, both of the emulsions may be considered to be safe with respect to the effect on skin pH values. With the help of ANOVA test, it was found that no effect on the pH values was produced by the active formulation **(Table 4.19.)**, and with the help of paired sample t-test it was found that there was no significant variation in the skin pH values when the base and active formulation were compared.

4.2.6.6. Skin Elasticity

Proteins named collagens are present in the dermis. Most of the collagens are present in bundles running horizontally through the dermis which are burried in a jelly like material called ground substance. Collagens account for upto 75% of the weight of the dermis, and are responsible for the resilience and elasticity of the skin. The collagen bundles are held together by elastic fibers. These are made of a protein called elastin. Despite their name, they are not involved in the natural elasticity of the skin.

Vitamin C is known to increase the collagen fibers of human skin (9, 192, 193, 222). In addition to vitamin C, Lipacide® has also been claimed to increase quantity and quality of collagen fibers (225). In the active formulation, both of the ingredients were used but there was no significant effect of formulation on the net elasticity of human skin in an application period of 4 weeks since no significant variation was determined by the ANOVA test **(Table 4.20.)**; and there was no significant difference between the two creams as determined by paired sample t-test. 4 week application period may be insufficient for the skin elasticity to be improved. Application period may be increased to investigate any improvement.

4.2.6.7. Panel Test

A questionnaire containing seven questions was prepared and the two copies of this form were given to each volunteer for sensory evaluation of the two

creams. Average points were calculated from the points assigned by each volunteer for each question for both of the creams and the results are shown in **Table 4.14.** and in **Figure 4.42.**

Average points for the first question, i.e. ease of application of creams were found to be 4.46 and 4.36 for the active formulation and the base, respectively. This indicates that both of formulations can be easily applied on the skin. Average points regarding spreadability were 4.36 for formulation and 4.27 for base which means that the active formulation spreads on skin better than the base. Average points for feel on application were 3.91 for the active formulation and 3.64 for the base. This indicates that formulation is felt better on the skin than base. Average points for the sense in long-term application of creams were 4.18 and 4.27 for the active formulation and base respectively. This shows that base produces more pleasant feeling on application to skin than the active formulation. There was no irritation on the skin as both of the creams were assigned 0 points for irritation by all the volunteers. Shine on skin was 3.73 for the active formulation and 3.82 for the base. This is expected since the base contains higher quantity of oil than the active formulation. Similarly, the base leads to more softness of the skin than formulation. The average points for the base was 4.82 while it was 4.56 for the active formulation.

Conclusively, there was no big variation between the active formulation and the base regarding the sensory evaluation. Both of the creams behaved similarly from the sensory point of view.

As a conclusion of this study:

- a w/o/w emulsion containing two incompatible antiaging agents can be formulated using macadamia nut oil
- there was no phase separation at all storage conditions for a period of 6 months
- Average globule sizes of emulsions first decreased and then increased leading to coalescence
- pH values of the emulsions decreased with time
- liquifaction of emulsions increased with time
- viscosity of formulation decreased with time
- vitamin C at a concentration of 1 %, increased moisture of the skin
- the formulation had no effect on skin sebum content
- the formulation did not change the elasticity of skin
- the formulation did not change the pH of skin
- the formulation is safe as it did not produce any irritation
- the formulation had no effect on melanin
- stability of the multiple emulsion system formulated in this study has to be improved.

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