AN INVESTIGATION OF THE ANTIOXIDANT PROPERTIES OF SOME CULINARY HERBS AND THEIR POTENTIAL APPLICATION IN A COSMETIC PREPARATION

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I Nareshini Naidoo (20103350) and Prof. B. Odhav and Mr. K. Devchand do hereby declare that in respect of the following dissertation:

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Ms. Nareshini Naidoo

DEDICATION

"This thesis is dedicated to my dearest parents whom have been my strongest support system during my studies. Their encouragement and motivation was the driving force in providing me with the power to attain all of my achievements thus far in my life: both academically and personally. Despite all of the challenges and obstacles that I have faced in my academic progress, they have never allowed me to give up and loose faith in myself."

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LIST OF ABBREVIATIONS

AIV	- antioxidant index value
AOM	- Active oxygen method
AR	- antioxidant response
BHA	- butylated hydroxyanisole
BHT	- butylated hydroxytoluene
CAIV	- cumulative antioxidant index value
GRAS	- Generally recognized as safe
HPLC	- High Performance Liquid Chromatography
IP	- Induction period
IRAC	- International Agency for research in cancer
MPA	- metaphosphoric acid
NO	- nitric oxide
PC	- phosphadycholine
PE	- phosphatidylethonalomine
PG	- propyl gallate
PS	- phosphatidylserine
ROS	- reactive oxygen species
TBA	- Thiobarbituric acid

ABSTRACT

Several herbs are well known for their nutritional applications. The reported study tested some herbs for their efficacy as antioxidants. The herbs and combinations thereof were analyzed for antioxidant activity using two comparative methods: the beta-carotene bleaching method and through measurement by the Rancimat apparatus. The efficacy of the antioxidants was also tested in a cosmetic base cream. The concentration of the natural antioxidant vitamins, such as vitamin C, vitamin E and beta-carotene was determined by analytical methods. The methods used for analysis provided adequate results for interpretation.

All herbs exhibited antioxidant activity, at comparable levels. The two methods used for analysis showed variable results, as previous researchers have shown. In terms of antioxidant potency, the herbs were ranked as follows: fenugreek > coriander > oregano > sage (beta-carotene bleaching method), oregano > sage > coriander > fenugreek (Rancimat method). Oregano and sage were good synergists, when combinations of herbs were used. Fenugreek, unlike the other herbs under investigation, is a prooxidant when used at higher concentrations. A direct relationship was observed between the concentration of herbs and antioxidant potency for sage, oregano and coriander. Fenugreek which is prooxidant at higher concentrations, should be used as an antioxidant independently rather than a synergist. This was the most important finding in the reported study. Similar antioxidant activities of the herbs were observed in the base cosmetic formulation.

The reported study has provided an adequate base for further quantitative research into the innovative topic of antioxidants.

CHAPTER 1

INTRODUCTION

In the personal care industry, the category of skin care treatment is the most dynamic and exciting of all. Since appearance reveals ones age, traditional skin care manufacturers have for years taken on the task of finding ways, primarily through the use of topical cosmetics to smooth wrinkles and bring back the look of youth with a range of skin care products (Madley, 2000). Intervening the normal aging process has become second nature to most women, and more recently with men, and is facilitated by the continuous stream of new anti-aging products arriving on the market (Knowlton, 2001). There are currently two major categories that are receiving attention in the skin care market: natural antioxidant extracts and functional herbs/botanicals.

Herbs and plants have already proved useful as a tool in complementary medicine. Folkloric use of botanicals was relatively unsophisticated, found usually in the form of infusions, poultices and compresses as well as vinegar, distillates and wines. Only in the last century did it become possible for research chemists to scientifically test, measure and record the effects of plant extracts on the human body, and find new ways of enhancing natural products through the use of sophisticated equipment and testing procedures. Today, holistically balanced and standardized extracts can be reproduced in identical batches, with great emphasis placed on the extract's final efficacy when incorporated into finished cosmetic products.

The sudden interest in herbal cosmetics stems from the market recognition that consumers are interested in natural approaches to skin care (Madley, 2000). However, this "back to nature" approach to skin care is still in its infancy, because scientific knowledge of specific botanicals varies widely (Madley, 2000). As a result, there is a need for research to be conducted in the field of natural ingredients, which could replace the harsh synthetic compounds that have customarily, been used in cosmetic preparations.

The presence of free radicals in a biological system containing polyunsaturated fats, and other material susceptible to oxidative destruction is potentially harmful because the damage associated with the initial production of the radicals can be greatly amplified through a chain mechanism involving peroxyl radicals. Although the chain reaction eventually becomes self-terminating when two peroxyl radicals combine to give non-radical products, there may still occur a massive amount of destruction of the unsaturated lipid, depending on the chain length. Chain reactions can be strongly inhibited by the addition of a small amount of a chain-breaking antioxidant.

Primary antioxidants are usually compounds that donate a hydrogen atom to lipid free radicals to produce a relatively stable radical, which is poor at propagating the chain reaction and hence interrupts the autoxidation process. Antioxidants of this type are mainly phenolic in structure and include tocopherols - gallic acid and derivatives, flavonoids-including carnosine, rosmaric acid and many other natural components.

Secondary antioxidants are components that have no antioxidant action in pure fats but are effective in the presence of other chemical minor components, either by improving the effectiveness of primary antioxidants or by inhibiting the effect of prooxidants. Natural antioxidants of this type include phospholipids, which have a synergistic action with primary antioxidants such as tocopherols.

There have also been reports indicating a sharp difference in the quality between synthetic and natural antioxidants. Much ~~A lo t of~~ work has been done to show that natural vitamin E is more bioavailable or functions at a higher level than synthetic vitamin E (Frei, 1994). A wide range of natural sources has been shown to contain antioxidants. These include plant extracts, herbs and spices, and fermentation products. Interest in natural antioxidants in the food industry and in medicine also stems from their potential health benefits. Many of these compounds have been associated with therapeutic properties and in certain instances have been shown to possess anticarcinogenic, antimutagenic, and cardioprotective activities. Reviews of the literature on natural antioxidants from plant extracts and spices have been determined by a wide

range of testing methods, however the conditions of oxidation make it increasingly difficult to make a realistic assessment of the efficacy of various natural antioxidants. Furthermore, antioxidant activity of natural antioxidants depends to a large extent on the mode of extraction, presence of inhibitors, nature, and concentrations of active components (Pokorny 1994).

Modifications to the skin's cutaneous tissue occurring over time have two origins: chronological aging, referring to genetically programmed processes; and extrinsic aging or photoaging, related to outside aggressions like ultra violet (UV) radiation, environmental pollutants and heavy metals (Benoit *et al*., 2000). Tissue damage owing to such environmental stimuli results from various oxidative processes, and this so-called "oxidative stress" involves a large number of chemical reactions and very diverse oxidizing species (Carletto and Nicolay, 2000). These aggressions trigger the accumulation in the skin of reactive oxygen species that can cause premature aging (photoaging) and carcinogenesis.

The aging process exemplifies the cumulative result of free radical damage to cells, tissues and organs. The human body has built in defense mechanisms for counteracting free radical reactions, however the aging process, disease, or both (Majeed *et al.*, 1996) can overwhelm the antioxidant defense system. As medical science gradually unravels the mysteries of skin aging, so are the cosmetic chemists who are constantly trying to catch up with creams, serums and lotions that they hope will help to turn back the clock. A primary focus for anti-aging cosmetics right now should be the antioxidant route. Applications of such cosmetic products can limit photoaging and skin cancer, which are both a growing concern to the dermatological and cosmetic industries, as well as to the consumer.

A major group of naturally occurring antioxidants includes the antioxidant vitamins viz. vitamin C, vitamin E and beta-carotene. In the last decade nutrition science has made significant advances in understanding the various roles of antioxidant vitamins in human diets. While antioxidant vitamins have for many years been important in polymer and

food sciences, recent research indicates that they may also be of key importance in life processes. Advances in the medical and nutritional sciences have clearly shown the benefits of consuming certain types of foods, particularly leafy green vegetables and certain fruits, which contain the above mentioned nutrients against a number of chronic diseases (Pokorny 1994).

The research reported had aimed in determining the extent of the antioxidant effect of culinary herbs, and the subsequent cumulative antioxidant effect in a base cosmetic preparation. The objectives were thus, firstly, to measure/determine the levels of antioxidant vitamins (vitamin C, vitamin E and beta-carotene) in the culinary herbs: sage, oregano, coriander and fenugreek. The second objective was to qualitatively evaluate the antioxidant effect of active antioxidant components present in the herbs. The evaluation methods of choice were firstly, the measurement of antioxidant response via a beta-carotene bleaching method and secondly, the measurement of antioxidant index via the Rancimat apparatus which measures induction periods. These methods were also used to determine complementary and synergistic antioxidant effects of the herbs, by evaluating effects of various combinations of the herbs, which was the third objective. The fourth and final objective was to determine the cumulative antioxidant effect of the herbs in a base cosmetic preparation.

The research reported could have commercial applications for both the food and cosmetic industries, as it could offer elucidation to some of the problems that exist in understanding the interaction of antioxidant vitamins. This research also reported an efficient method for measuring antioxidant activity. Furthermore, it recommended some natural herbal components as replacements to synthetic/chemical components.

CHAPTER 2

LITERATURE REVIEW

2.1 THE "BACK TO NATURE" APPROACH

"Nature is proving that she can't be beaten- not by the likes of us." Never were the words of Robert E. Sherwood more apt than in the 21st century, when despite the continual destruction of earth's natural resources, nature persists by exceeding the most sophisticated man-made products.

Herbs were once an essential part of people's lives, as flavourings for foods, remedies for ailments and their symptoms, and as aids to beauty. Of the three usages's, only the first had continued into modern times (Craig and Harris, 1998). However, disenchantment with conventional medicine and concern for the well being of the planet and all life forms on it has encouraged people to look for more natural, harmonious and less harmful ways of living, and the long forgotten uses of herbs are once more being explored. Furthermore there is increasing concern about the substances that go into all kinds of commercial products-from foods to medicinal drugs- and their side effects (Craig and Harris, 1998). The trend towards more traditional and natural ways of doing things that are healthy and do not harm the environment or other living creatures is evident in the foods we eat, the medicines we take, the cosmetics we use, which seems to lead inevitably to the use of herbal products in the home.

2.2 APPLICATIONS OF FUNCTIONAL HERBS/BOTANICALS

The use of herbs for medicinal properties is widespread amongst the various conventional and non-conventional therapies such as alternative medicine, herbal medicine ~~simply means any approach to solving a health problem that's different from those used by conventional practitioners of western~~, homeopathy, aromatherapy, and ayurvedic medicine. Many herbal components are functional ingredients in many toiletries, detergents and of course cosmetics.

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2.2.1 Functional herbs/botanicals in cosmetics ~~Functional herbs/botanicals in cosmetics~~

From skin, body and hair care products to fragrances and cosmetics, the market has demonstrated a consumer trend towards using naturally derived products. Despite the sophisticated technologies used to produce modern cosmetics, the formulation for many of our health and beauty remedies today is based on traditional techniques that have been handed down through the generations (Earle 1994). In fact, modern scientific research is now endorsing much of the folklore surrounding natural therapies that have been used for centuries (Earle, 1993). However, only in the last century did it become possible for research chemists to scientifically test, measure and record the effects of plant extracts on the human body, and find new ways of enhancing natural products through the use of sophisticated equipment and testing procedures (Franklin, 2000). Through raw material suppliers, manufacturers have access to a wide range of natural cosmetic ingredients, from essential and vegetable oils to botanical extracts and natural actives. The ingredients range from extracts from a single plant species to complex combinations, offering endless permutations of types and activities (Franklin, 2000).

In addition to the basic range of raw materials needed to make preparations to cleanse and protect our skin and hair, there is also a range of natural plant extracts that can be added to give products specific benefits (Earle, 1993). For example, a skin tonic may be created to suit a dry, sensitive skin by adding chamomile flowers, or a product may be made suitable for an oily, combination skin by including nettles and yarrow (Earle, 1993).

Plant extracts are among the favorites of modern skin and hair care products, as those valuable substances provide ideal care and effective protection for skin and hair (Franklin, 2000). Fruit extracts play a significant role in many plant-based ranges, with tropical or exotic fruit featuring prominently. The constituents of mangoes, kiwis,

coconuts and papaya are well known for their regenerating, caring and moisturizing effects on the skin and hair, as well as their vitamin and mineral content (Franklin, 2000). Bearberry is a functional ingredient that not only inhibits the progress of skin darkening, but also effectively reduces existing pigmentation (Tew, 2002). Plant extracts like lady's mantle, lady's thistle, horsetail and wheatgerm extract provide hydration and help to regulate moisture content (Tew, 2002). Essential oils of neroli, geranium, lavender, jojoba and sandalwood calm, soothe, pamper and moisturize skin (Tew, 2002). Horsetail, yarrow, nettle and houseleek are among the astringent, cleansing herbs that will contract dry, oily skin (Garland, 1998). Juniper, sage, thyme, rosemary and clove are bactericidal, therefore controlling bacteria that cause blemishes (Daswani, 1996). Yeasts and beech tree extracts are rich in amino acids and minerals, which promote skin respiration and oxygen uptake (Tew, 2002). Vanillin is a functional ingredient in skin lightening products (Tew, 2002). Saffron is a special herb for nourishing the skin (Frawley, 1989). *Ginkgo biloba*, used frequently in herbal supplements for its effects on brain function and circulation, also features widely in energy boosting products in the skin and hair (Franklin, 2000).

The natural skin whitening agent, *Rumex occidentalis*, is a liquid extract of the Canadian Rumex species (Tew, 2002). Brazil nut and soya have demonstrated good potential in hair care applications (Franklin, 2000). A natural anti-irritant has been derived from oats (Hughson, 2000), making it suitable for products for sensitive skin and anti-ageing products that otherwise tends to produce minor irritations. The *Naix de Grenoble* walnut has been shown to have biostimulating properties (Hughson, 2000). *Shorea robusta*, an Indian tree rich in tannins is used in the treatment of large pored and greasy skin and also possesses astringent action (Daswani, 1996). An extract from cakile, a hardy desert plant that can withstand extreme environmental conditions. The extract which is said to retain 1000 times more water than any other known natural extract, enables skin cells to capture and hold moisture enhancing water circulation and toxin elimination, promoting optimal cell functioning and stimulating the skins natural barrier (Tew, 2002). Henna and mint have been used in hair loss products (Daswani, 1996). Ayurvedic turmeric cream is used for acne and improving complexion (Frawley, 1989).

2.3 LIPID AUTOXIDATION

Lipids form one of the major bulk constituents in food and biological systems. This group of organic biochemicals occurs in plants and animals either as storage lipids, which are potential sources of energy by beta-oxidation, or as membrane lipids (Jadhav *et al*., 1996). Storage lipids are triglycerides, whereas membrane lipids include phospholipids, sterols, sphigolipids and glycolipids (Jadhav *et al*., 1996). Many plant foods contain highly unsaturated lipids while lipids of animal origin have lower levels of unsaturated lipids, but contain certain amounts of higher unsaturated fatty acids (Madhavi *et al*., 1996). Unsaturation in fatty acids makes lipids susceptible to oxygen attack leading to complex chemical changes that eventually may manifest themselves in the development of free radicals (Jadhav *et al*., 1996). The process of free radical formation is known as autoxidation. Figure 2.1 depicts a schematic representation of the overall process of lipid oxidation.

Lipis oxidation has long been recognized as a major problem in the storage of fatty foods. Oxidative changes can result in repugnant flavours, in destruction of valuable nutrients and even in generation of toxic compounds. The better understanding of lipid oxidation achieved has assisted in the answering of questions of importance in food systems.

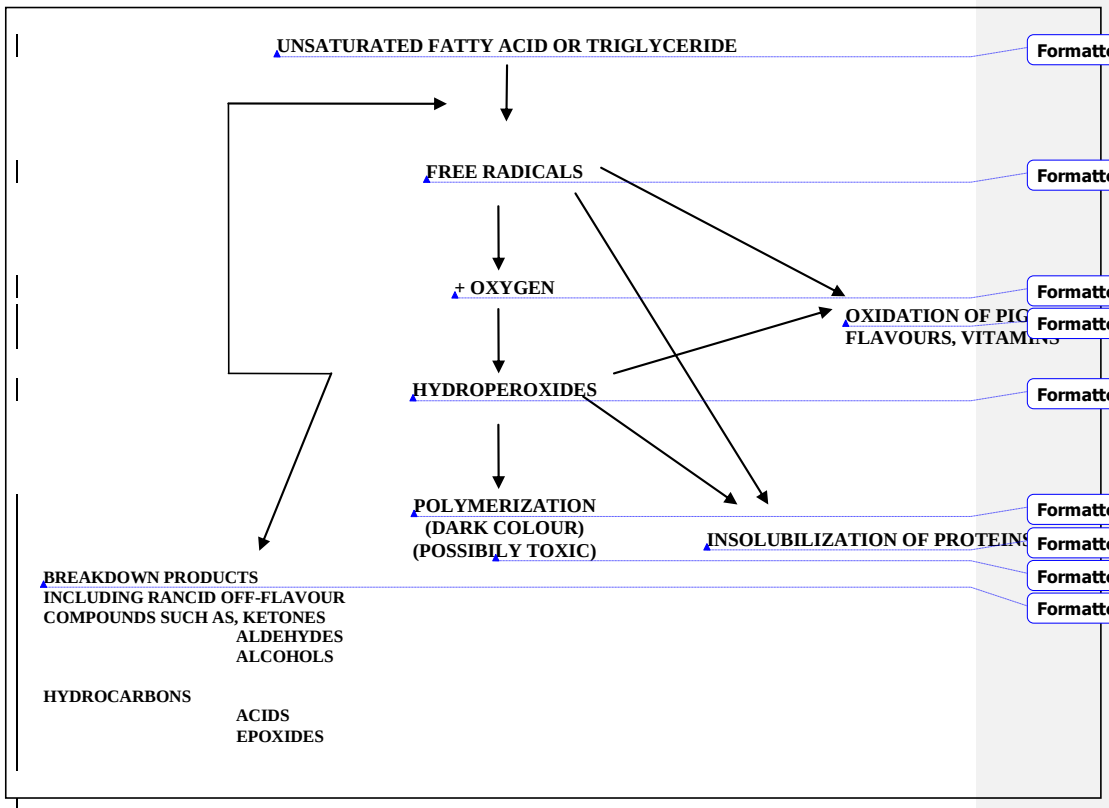

Figure 2.1: Overall mechanism of lipid oxidation (Madhavi et *al.*, 1996)

2.3.1 The autoxidation of unsaturated fatty acids

The autoxidation of unsaturated fatty acids can be divided into three phases: initiation, propagation and termination (Schuler, 1980). In the first phase, initiation: initiators, such as energy (light, heat), traces of heavy metals, and peroxides attack the substrate RH and produce highly reactive free radicals ($R^{\cdot}$) - Eq. (1) (Schuler 1980). Initiation reactions take place either by the abstraction of a hydrogen radical from an allylic methylene group of an unsaturated fatty acid or by the addition of a radical to a double bond (Jadhav *et al.*, 1996).

$$RH \longrightarrow R^{\cdot} + H^{\cdot} \quad (1)$$

$$ROOH \longrightarrow RO^{\cdot} + HO^{\cdot} \quad (2)$$

$$2ROOH \longrightarrow RO^{\cdot} + ROO^{\cdot} + H_2O \quad (3)$$

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Also, lipid hydroperoxides, which exists in trace quantities prior to the oxidation reaction, breaks down to yield radicals as shown by Eqs. (2) and (3) (Jadhav *et al*., 1996). Various pathways including the reaction of singlet oxygen with unsaturated lipids or the lipoxygenase-catalyzed oxidation of polyunsaturated fatty acids (Jadhav *et al*., 1996) forms lipid hydroperoxides.

In the propagation phase, free radicals react with oxygen to produce peroxide radicals ($ROO^{\cdot}$) – Eq (4) (Schuler, 1980). Peroxide radicals have the ability to attack another fatty acid $RH^{\cdot}$. The result is a peroxide (ROOH) and a new free radical – Eq (5) (Schuler, 1980). Thus a general feature of the reactions of free radicals is that they tend to proceed as chain reactions, i.e. one radical propagates another and so on (Jadhav *et al*., 1996).

$$R^{\cdot} + 3O_2 \longrightarrow ROO^{\cdot} \quad (4)$$

$$ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot} \quad (5)$$

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Lipid peroxy radicals ($ROO^{\cdot}$) initiate a chain reaction with other molecules, resulting in the formation of lipid hydroperoxides and lipid free radicals (Jadhav *et al*., 1996). This reaction, when repeated many times, produces an accumulation of hydroperoxides (Jadhav *et al*., 1996). The oxygenation reaction (4) is very fast, and requires no activation energy, and therefore the concentration of $ROO^{\cdot}$ is much higher than that of $R^{\cdot}$ in most food systems where oxygen is present (Gordon, ~~1990~~1996). This propagation reaction becomes a continuous process as long as unsaturated lipid or fatty acid molecules are available (Jadhav *et al*., 1996). Peroxides are unstable compounds, which decompose into radicals such as, aldehydes, ketones and alcohols (Schuler, ~~(~~1980). These volatile

decomposition products are responsible for the off-flavour often observed in many food products (Schuler, 1980).

In the termination phase, the quantity of highly reactive compounds rises constantly until they begin to interact (Schuler, 1980). Stable deterioration products are subsequently formed from these reactions (Schuler, 1980). When there is a reduction in the amount of unsaturated lipids (or fatty acids) present, radicals bond to one another, forming a stable non-radical compound (Jadhav *et al.,* 1996). Then the concentration of radicals and peroxides falls.

$R^{\cdot} + R^{\cdot} \longrightarrow R - R$ (6)

$ROO^{\cdot} + R^{\cdot} \longrightarrow ROOR$ (7)

$ROO^{\cdot} + ROO^{\cdot} \longrightarrow ROOR + O_2$ (8)

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Thus, the termination reactions lead to interruption of the repeating sequence of propagation steps of the chain reaction (Jadhav *et al*., 1996). Radical coupling results in the release of energy equivalent to the strength of the bond being formed (Jadhav *et al*., 1996). Subsequently, the released energy is dissipated as heat.

2.3.2 Free radicals

A free radical is any species capable of independent existence (hence the term "free") that contains one or more unpaired electrons. An unpaired electron is any electron that is alone in an orbital. A non-radical (Deshpande *et al*., 1996) can be generated by the loss of a single electron from a non-radical or by the gain of single electron from free radicals. The presence of one or more unpaired electrons causes the species to be paramagnetic and sometimes makes it highly reactive (Deshpande *et al.,* 1996). The simplest free radical is an atom of the element hydrogen, with one proton and a single electron (Halliwell, 1994). Free radicals are electrically neutral, and solvating effects are generally

very small (Jadhav *et al*., 1996). They are considered to be bonding-deficient and hence structurally unstable (Jadhav *et al*., 1996). Radicals, therefore, tend to react whenever possible to restore normal bonding. That is why a free radical is highly reactive (Jadhav *et al*., 1996).

Reactive oxygen species are produced continuously in the human body as a consequence of normal metabolic processes (Halliwell 1995). If free radicals are not inactivated, their chemical reactivity can damage all types of cellular macromolecules, including proteins, carbohydrates, lipids and nucleic acids (Langseth, 1995). The chemical reactivity of free radicals varies; one of the most reactive is the hydroxyl radical ($OH^{\cdot}$). Hydroxyl radicals react at a diffusion-controlled rate with almost all molecules in living cells (Halliwell, ~~1995~~1994). The reactions of the hydroxyl radical can be classified into three main types: hydrogen abstraction (e.g. reactions with alcohols and phospholipids), addition (e.g. reactions with aromatic ring structures such as with the purine and pyrimidine bases present in DNA and RNA), and electron transfer (e.g. reactions involving organic and inorganic compounds (Deshpande *et al*., 1996). Hence, when $OH^{\cdot}$ is formed *in vivo*, it damages whatever it is generated next to, as $OH^{\cdot}$ cannot migrate any significant distance within the cell. It therefore attacks proteins, carbohydrates, DNA and lipids (Halliwell, 1995).

Superoxide radical ($O_2^{\cdot -}$) is the electron reduced product of oxygen. This radical can emanate from so called "accidents of chemistry" (Halliwell, 1995). It may be generated by "autoxidation" reactions, in which compounds such as catecholamines, tetrahydrofolates, and reduced flavins react directly with O_2 to form $O_2^{\cdot -}$. The $O_2^{\cdot -}$ then oxidizes more of the compound and sets up a free radical chain reaction (Halliwell, 1995).

Reactivity depends on the radical and what the radical is presented with. If two free radicals meet, they can join their unpaired electrons to form a covalent bond. A more biologically relevant example is the very fast reaction of $NO^{\cdot}$ and $O_2^{\cdot -}$ to form a non-radical product, peroxynitrite (NO_3^-) (Halliwell, ~~1995~~1994). However when a free

radical reacts with a non-radical, a chain reaction is set up (Halliwell, ~~1995~~1994). For example, an attack of reactive radicals upon fatty acid side chains in membranes and lipoproteins can abstract hydrogen, leaving a carbon centered radical and initiating the process of lipid peroxidation (Halliwell, 1994). When $OH^{\cdot}$ is generated adjacent to DNA, it attacks both the deoxyribose sugar and the purine and pyrimidine (Halliwell, 1994).

2.3.3 Defenses against oxidants

Autoxidation is an irreversible process of deterioration. It is not possible to prevent it completely, it can only be retarded (Schuler, 1980). Several factors can be manipulated to retard oxidation: input of energy has to be at a minimum, contact with traces of heavy metals have to be avoided, oxygen has to be substantially excluded and radicals have to be intercepted (Schuler, 1980). The initiator energy-light and direct heat is best excluded by storage of fat in a cool, dark place (Schuler, 1980). This is always the first preventative measure. Heavy metal traces are important initiators. Apparatus made from iron or copper are therefore not recommended (Schuler, 1980). Traces of such metals can often be rendered harmless by combining them chemically with complexing agents such as citric or tartaric acids, EDTA, lecithin, etc (Schuler, 1980). Contact of oxygen has to be avoided. Residual or penetrating oxygen can be removed by oxygen scavengers, which convert oxygen into a harmless form (Figure 2.2) (Schuler, 1980). A good example of a scavenger is ascorbic acid. Combining these with agents known as radical scavengers can inactivate radicals. Elimination of radicals interrupts the chain reaction. In the first phase radical scavengers donate hydrogen atoms to free radicals (Schuler, 1980).

$$InH + RO_2 \text{ (or } R^{\cdot}) \longrightarrow RO_2H + In^{\cdot} \text{ (or } RH + In) ,$$

in which $In^{\cdot}$ is the inactive radical

Additionally, they can be combined directly with radicals to form inert products (Schuler, 1980). Oxygen scavengers and radical scavengers are commonly called antioxidants.

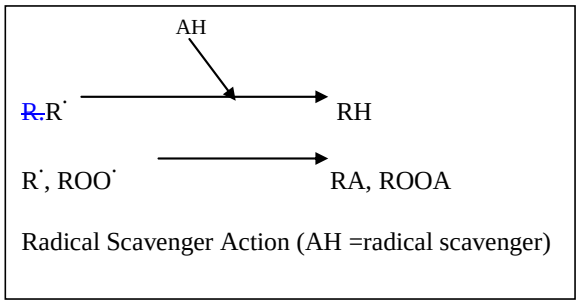

Figure 2.2: Schematic representation of radical scavenging reactions

2.3.3.1 Antioxidants

All antioxidants are classified into two groups, namely primary antioxidants or chain-breaking antioxidants, which can react with lipid radicals to convert them to more stable products and secondary or preventive antioxidants which reduce the rate of chain reaction by a variety of mechanisms (Gordon, 1996). A molecule will be able to act as a primary antioxidant if it is able to donate a hydrogen atom rapidly to a lipid radical and if the radical derived from the antioxidant is more stable than the lipid radical, or it is converted to other stable products (Gordon, 1996). Primary antioxidants may either delay or inhibit the initiation step by reacting with a free radical or inhibit the propagation step by reacting with the peroxy or alkoxy radicals:

$AH + L^{\cdot} \longrightarrow A^{\cdot} + LH$

$AH + LOO^{\cdot} \longrightarrow A^{\cdot} + LOOH$

$AH + LOO \longrightarrow A + LOH$ (Rajalakshmi and Narasimhan 1996).

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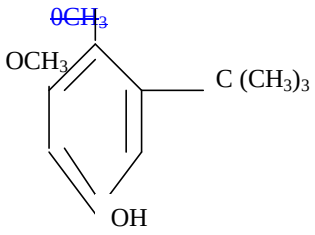


FFigure 2.3: Chemical structure of BHT

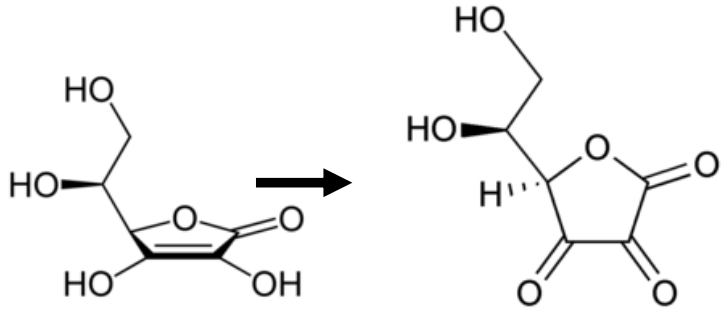
Examples of primary antioxidants include hindered phenols such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) (Figure 2.3) and alpha-tocopherol (Frankel, 1991). Many of the naturally occurring phenolic like flavonoids, eugenol, vanillin, and rosemary antioxidant also have chain-breaking properties (Rajalakshmi and Narasimhan, 1996). To be effective, these compounds must compete with the unsaturated lipid substrate, which is normally present in the highest concentration (Frankel, 1991). Primary antioxidants are effective at very low concentrations, and at higher levels they may become proxidants (Rajalakshmi and Narasimhan, 1996).

Compounds which retard the rate of autoxidation of lipids by a process other than that of interrupting the autoxidation chain by converting free radicals to more stable species are termed secondary antioxidants (Gordon, 1996). These may operate by a variety of mechanisms including binding metal ions, scavenging oxygen, decomposing hydroperoxides to non-radical species, absorb UV radiation or deactivate singlet oxygen (Gordon, 1996). Secondary antioxidants usually only show antioxidant activity if a second minor component is present in the sample (Jadhav *et al*., 1996). This can be seen in the case of sequestering agents which are effective in the presence of metal ions, and reducing agents such as ascorbic acid which are effective in the presence of tocopherols or other phenolic antioxidants (Gordon, 1996). Typical examples of secondary antioxidants are therefore sequestering agents, metal ions, reducing

OH

agents (ascorbic acid), and tocopherols or other phenolics (Jadhav *et al.*, 1996).

Ascorbic acid is oxidized to dehydroascorbic acid (Figure 2.4) when it functions as an oxygen scavenger (Madhavi *et al*., 1996). The oxygen scavenging activity of ascorbic acid ~~make~~makes it very useful in canned or bottled products with a headspace of air but in fatty foods ascorbyl palmitate is often more effective as an antioxidant because of its increased solubility in the fat phase (Gordon, 1996). Citric acid (Figure 2.5) is another example of a secondary antioxidant: it is very effective in retarding the oxidative deterioration of lipids in foods and is commonly added to vegetable oils after deodorization (Gordon, ~~1990~~1996). It is also very efficient in reducing the proxidant effect of a range of metals (Gordon, ~~1990~~1996).



Ascorbic acid **Dehydroascorbic acid**

Figure 2.4: Oxidation of ascorbic acid

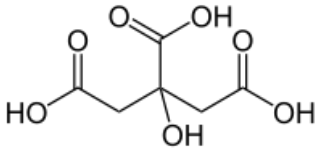


Figure 2.5: Chemical structure of citric acid

Other example of secondary antioxidants includes the quinones such as ubiquinone and alpha-tocopheroquinone, which must compete with oxygen in the fast reaction. These compounds may therefore only be active in biological systems where the oxygen pressure is relatively low (Frankel, 1991).

Antioxidant synergism is a process by which the antioxidant effect of multi-component systems is reinforced. Significant synergism is generally observed between free radical acceptor antioxidants and metal chelators (Frankel, 1991). Synergistic antioxidants can be broadly classified as hydrogen donors to the phenoxy radical, thereby regenerating the primary antioxidant (Rajalakshmi and Narasimhan, 1996). Hence phenolic antioxidants can be used at lower levels if a synergist is added simultaneously to the food product (Rajalakshmi and Narasimhan, 1996). Antioxidant synergism is particularly important in natural tocopherols found in soybean oil and metal chelators, such as citric acid, which are essential to ensure oxidative stability (Frankel, 1991). The synergism between 3-BHA and BHT is schematically represented in Figure 2.6.

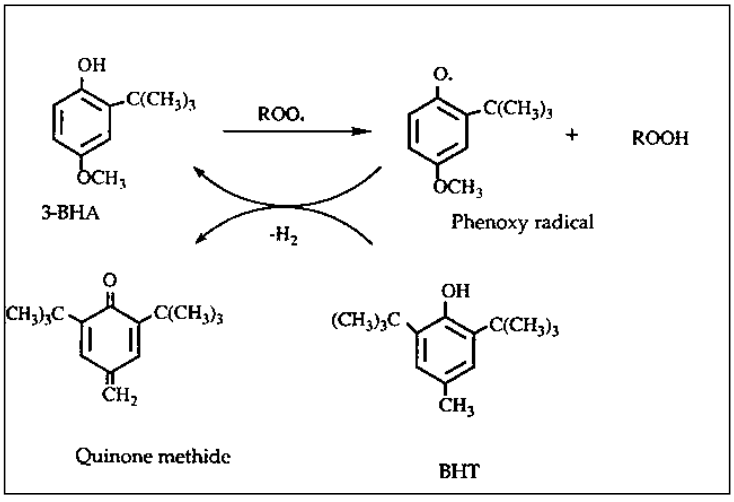


Figure 2.6: Synergism between 3-BHA and BHT (Madhavi *et al.*, 1996)

2.4 METHODS TO EVALUATE ANTIOXIDANTS

Although the literature on stability evaluations of food lipids is extensive, the published data comparing the effectiveness of various antioxidants are often difficult to interpret because of the debatable methodologies, particularly the choice of methods utilizing inappropriate oxidation conditions (Frankel, 1993). Essential to testing the effectiveness of antioxidants is a means of determining the induction period and measuring the rate of oxidation (Ross, 1984). A desirable method for evaluating the antioxidant activity of a compound should be rapid, reproducible, should require small amounts of chemicals and should not be influenced by the physical properties of the compound (Marco, 1968).

Natural antioxidants have been especially difficult to evaluate because of the use of crude extracts and the occurrence of complex interfacial phenomena in oils and food emulsions (Frankel, 1993). Thus, interpretation of data pertaining to lipid oxidation should take into account the limitations of the methodology used (Frankel, 1993). The degree of lipid oxidation can be measured by chemical and/or physical methods as well as stability tests, which measure the stability of oil under conditions that attempt to accelerate the normal oxidation processes (Gray, 1978). Burkow and colleagues (1995) found in their investigations on cod liver oil, that the ranking of antioxidants varied considerably depending on the methods used, and that increasing the temperature seemed to decrease the usefulness of the method. An antioxidant evaluation has to be done with as many methods as possible and under conditions relevant for normal storage and use (Burkow *et al*., 1995). Meyer (1994) had previously proposed the development and use of a wider range of standard model systems for testing new antioxidants, i.e. the use of a range of model systems of different hierarchical levels of complexity, where the highest, most complex level closely resembles the selected product requiring oxidative protection.

The induction period (IP) is measured as the time required to reach an endpoint of oxidation corresponding to either a level of detectable rancidity or a sudden change in the

rate of oxidation (Frankel, 1993; Presa Owens and Lopez-Sabater, 1995). Measurements of IP under standard conditions are generally used as an index of antioxidant effectiveness. For practical purposes however, predictions of oxidative stability in foods and oils based on measurements of IP should be related to measured product shelf life (Frankel, 1993). Accelerated oxidation tests should be calibrated for each formulation, and the conditions used should be maintained as close as possible to those under which the food is stored (Frankel, 1993).

2.4.1 Accelerated stability tests

To estimate the stability or susceptibility of a fat to oxidation, the sample is subjected to an accelerated oxidation test under standardized conditions and a suitable endpoint is chosen to determine signs of oxidative deterioration (Frankel, 1993). Several parameters (e.g. temperature, metal catalysts, oxygen pressure, shaking) are manipulated to accelerate oxidation and the development of rancidity in oils and emulsions (Frankel, 1993). Heating is the most common and effective means of accelerating oxidation. In the presence of antioxidants, the activation energy of lipid oxidation increases because antioxidants lower the rates of oxidation reactions by increasing the overall energy of activation (Frankel, 1993). The order of activity and ranking of different antioxidants depends on whether they are tested at high or low temperatures. Conventional stability tests are listed in Table 2.1.

Table 2.1: Standard accelerated stability tests (Source: Frankel, 1993)

Test	Conditions
Ambient storage	Room temperature
	Atmospheric pressure
Light	Room temperature
	Atmospheric pressure
Metal catalysts	Room temperature
	Atmospheric pressure
Weight-gain method	30-80^{o}C
	Atmospheric pressure
Schaal oven test	60-70^{o}C
	Atmospheric pressure
Oxygen uptake	80-100^{o}C
	Atmospheric pressure
Oxygen bomb	99^{o}C
	65-115 psi O_2
Active oxygen method (AOM)	98^{o}C
	air bubbling
Rancimat	100-140^{o}C

Although testing stability under ambient conditions may approximate real storage conditions of foods, the procedure is too slow to be of practical value (Frankel, 1993). Furthermore, under slow oxidation, the reproducibility of results is compromised by many variables that are difficult to control over prolonged storage periods (Frankel, 1993). Methods using light- and metal-catalyzed oxidation provide rapid screening tests. However, in the presence of sensitizers the mechanism of photo-oxidation is different from that of the free-radical autoxidation that usually occurs in foods (Frankel, 1993).

Photo-oxidation results in the formation of different flavour precursors with different volatile breakdown products and flavour significance (Frankel, 1993). The weight-gain method, based on the increase in weight due to oxygen absorption, is not very sensitive. The endpoint requires a level of oxidation that is beyond the point where flavour deterioration is detectable in polyunsaturated oils (Frankel, 1993). The Schaal Oven test, run at 60-70^{o}C, has fewer limitations associated with it. The endpoint represents a lower degree of oxidation and results correlate well with evaluations of actual shelf-life (Frankel, 1993).

The Schaal Oven Test and the Active Oxygen Method (AOM) are the oldest and most widely used. These tests are of great utility when comparing oils for their general stability against oxidation. In the Schaal Oven Test, samples of fat are stored at raised temperature in open dishes. The measure of the degree of oxidation is the peroxide value, which starts to increase rapidly at the end of the initiation phase (Schuler, 1980). The AOM/Swift test is based on intense contact between the substrate and oxygen (Schuler, 1980). A constant stream of air is blown through the hot oil, and the peroxide value is measured at regular intervals (Schuler, 1980). Their major disadvantage is that they fail to predict stability, particularly the onset or nature of off-flavours, in lipid-containing products. Three reasons may be advanced for these limitations: i.) Food products are often formulated as emulsions and the course of oxidation may be reasonably expected to be different than in a bulk oil; ii.) many food emulsions, such as salad dressings, are acidic and acids are known to catalyze the decomposition of peroxides, and iii.) food products are stored at ambient or at cold temperatures for long periods of time. If the mechanisms of oxidation change with temperature, lack of predictive ability from accelerated methods at high temperatures can be expected (Hassenhuetti and Wan, 1992). These conventional AOM test is also time consuming and expensive to run because it requires repeated titrations for peroxide values (Akoh, 1994).

Recently, the Metrohm Rancimat (Metrohm Instruments, Herisau, Switzerland) has been advanced as a method to determine resistance of an oil towards oxidation (Hassenhuettii and Wan, 1992). The Metrohm Rancimat developed as a rapid automated method,

produces similar results as the AOM (Laubli and Bruttel, 1986; Gordon and Mursi, 1994). In this test, the oil sample is placed in a vessel, subjected to an above-ambient temperature while oxygen is bubbled through to accelerate the oxidative process into its final steps where short-chain volatile acids are produced (Mendez *et al*., 1997). In a separate vessel, the acids formed are immediately dissolved in distilled water, and the conductivity of this solution is monitored at ambient temperature (Mendez *et al*., 1997). The products formed in the accelerated tests include volatile dicarboxylic acids, which contribute to the change in electrical conductivity in the Rancimat test (Gordon and Mursi, 1994). These products differ from those formed under normal storage conditions, which mainly comprise hydroperoxides that are detected in the traditional peroxide value test (Gordon and Mursi, 1994). The Rancimat method, however is considerably less costly and labour intensive than the AOM test (Laubli and Bruttel, 1986). An advantage of the Rancimat technique is that it is a continuous measurement, which requires no periodic analytical determinations, therefore, requires no organic solvents for titrations (Hassenhuetti and Wan, 1992). Gordon and Mursi (1994) reported in their studies, that the Rancimat method correlated well with stability under ambient storage conditions for a wide range of vegetable oils. Burkow and colleagues (1995) also reported on the reproducibility of the Rancimat method.

2.4.2 Lipid Oxidation Methods

The application of lipid oxidation methods involves measuring lipid oxidation after the sample has been oxidized under standardized conditions to a suitable endpoint (Frankel, 1993). Although sensory analysis is one of the most sensitive methods available, it is not practical for routine analyses and generally lacks reproducibility (Gray, 1978). The scoring by taste or odour panelists may vary greatly from laboratory to laboratory (Gray, 1978). Chemical and physical methods developed therefore attempt to improve reproducibility, sensitivity, and quantitativeness (Gray, 1978).

Analysis of volatiles by gas chromatography is closely related to flavour evaluation and is therefore the most suitable method for comparison with the results of sensory panel

tests (Frankel, 1993). The gas chromatography method can also provide useful data on the origin of flavour and odour volatiles and their precursors. Gas chromatography can be very useful in determining the extent of rancidity in pure mixtures such as vegetable oils (Gray, 1978). Gas chromatography was successfully used as a method to determine the inhibitory effect of antioxidants: alpha-tocopherol, beta-carotene and BHT on malondehyde formation from ω3 polyunsaturated fatty acids (Ogata *et al*., 1996). This method was also used as a method to determine the oxidation of n-3 fatty acids by measuring the headspace volatiles resulting from lipid oxidation (Boyd *et al*., 1992). However, in the case of more complex lipid systems such as foods, difficulties in identification and standardization will probably negate much of this usefulness (Gray, 1978).

Oxygen absorption methods have limited sensitivity and require high levels of oxidation as the endpoint for induction periods (Frankel, 1993). Determination of the peroxide value provides an empirical measure of lipid oxidation that is less sensitive and precise than sensory and headspace methods for volatiles (Frankel, 1993). The primary products of lipid oxidation are hydroperoxides, which are generally referred to as peroxides (Figure 2.7). Therefore, it seems reasonable to determine the concentration of peroxides as a measure of the extent of oxidation (Gray, 1978).

Although the peroxide value is a common measurement of lipid oxidation, its use is limited to the initial stages of oxidation. Since peroxides are vulnerable to further reaction, the complete oxidative history of the oil may not be revealed (Gray, 1978).

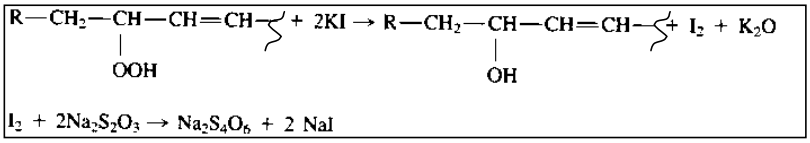


Figure 2.7: Principle of the measurement of peroxide values (Madhavi *et al*., 1996)

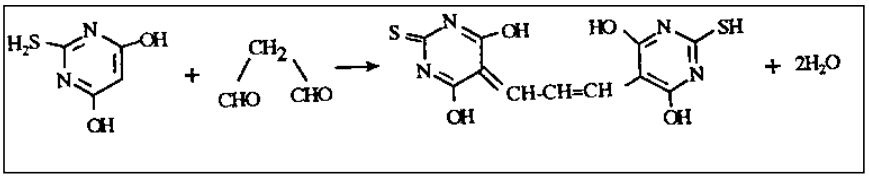


Figure 2.8: Principle of reaction for the measurement of TBA numbers (Madhavi *et al.*, 1996)

The thiobarbituric acid (TBA) method is based on the colour reaction between TBA and oxidation products of polyunsaturated lipids-Figure 2.8 (Frankel, 1993). The TBA test has been used since 1916 (Jacobson, 1993). This simple test is sensitive and very precise, but the information provided can be misleading. This is as a result of the test not being specific because many secondary oxidation products form TBA reactive substances (Frankel, 1993). Evidence that TBA can react with compounds other than those found in oxidizing systems to produce the characteristic red pigment has been presented in the literature (Gray, 1978). Despite the numerous pitfalls of the TBA test, many laboratories use it and good correlations can be developed between the TBA test and flavour scores (Jacobson, 1993).

The spectrophotometric method for carotene bleaching by co-oxidation of linoleic acid is simple and sensitive. The antioxidant comparisons can be based on minimizing beta-carotene loss in an emulsified; aqueous coupled oxidation of linoleic acid and beta-carotene (Marco, 1968). Frankel (1993) reports that this method may not be specific and is subject to interference from oxidizing and reducing agents present in crude extracts. In one study, the carotenoid decolouratisation reaction in organic medium was established as a unique thermodynamically compensated reaction, in which the source of energy (light or temperature) was able to remove electrons from the chromaphore and consequently destroy the colour of the pigment (Charleux, 1991). These authors also concluded that the carotenoid decolouration reaction mechanism is the same whether it is

promoted by enzymatic co-oxidation by fatty acid autoxidation, or simply by pigment oxidation. The beta-carotene bleaching method was employed to determine the antioxidant activity of sodium nitrite in meat (Zubillaga *et al.*, 1984), antioxidant properties of deferoxamine (Shimoni *et al*., 1994), antioxidant activity of flavonoid antioxidants in soybeans (Pratt, 1972; Pratt & Birac, 1979) and measured antioxidant activity of natural lipid antioxidants in chia seeds (Taga *et al*., 1984).

Gray (1978) reviews some other methods used less frequently to measure lipid oxidation: conjugated diene methods, fluorescence, infrared spectroscopy, polagraphy and refractrometry.

2.5 NATURAL ANTIOXIDANTS

In recent years, consumers and food manufacturers have been opting for products with "all natural" labels. The volume of such products increased 175% from 1989 to 1990, and the number of products claiming to be without additives or preservatives rose by 99% during the same period (Rajalakshmi and Narasimhan, 1996). Consequently, a lot of emphasis was given to the identification and incorporation of novel, natural antioxidants into food products. Mediating the low oxidative stability of lipids, through antioxidant addition, is important to health protection and for economic reasons (Marinova and Yanishlieva, 1994). The area of natural antioxidants developed enormously in the past decade mainly because of the increasing limitations on the use of synthetic antioxidants and enhanced public awareness of health issues (Rajalakshmi and Narasimhan, 1996). There has thus been a steady increase in the use of antioxidants for medical, nutritional and technical purposes (Lovaas, 1991). No assurance whatsoever can be given that a fraction of a compound isolated from natural sources is safe. Nevertheless, such an antioxidant would be a natural component of foods that we have consumed for many years (Whittern *et al*., 1984). Also, many natural antioxidants derive from renewable resources that will not be depleted when grown in a minimal input environment. Pokorny (1991) lists some of the advantages and disadvantages of natural antioxidants compared to synthetic antioxidants (Table 2. 2).

Table 2.2: Advantages and Disadvantages of Natural Antioxidants in Comparison with Synthetic Antioxidants

Advantages	Disadvantages
Readily accepted by the consumer, as considered to be safe, and not a "chemical".	~~1.~~ Usually more expensive if purified and less efficient if not purified.
No safety tests required by legislation if it is a component of a food that is "generally recognized as safe" (GRAS).	~~2.~~ Properties of different preparations vary if not purified.
	~~3.~~ May impart colour, aftertaste, or off-flavour to the product.
	~~4.~~ Safety often not known.

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Synthetic antioxidants such as BHT, BHA and propyl gallate (PG) have been widely used in many foods to prevent fat rancidity (Haumann, 1990). Recently, these synthetic substances have been shown to cause the following symptoms: enlarge liver size, increase liver microsomal enzyme activity, and convert some ingested materials into toxic or carcinogenic substances, especially if they are present in excessive amounts (Farag, Badei and Baroty, 1989). BHA has toxic and carcinogenic effects (Haumann, 1990). For example, dietary administration of BHT to rats caused fatal hemorrhages in the pleural and peritoneal cavities and in organs such as epitidymis testes and pancreas (Farag *et al*., 1989). BHA was identified as a carcinogen in laboratory animals by the International Agency for Research in Cancer in the United States (Haumann, 1990).

Many different types of natural antioxidants have been examined for their use as antioxidants, and some of them have been found to provide enhanced stability to lipids (Six, 1994). Many food ingredients contain antioxidant compounds (Table 2.3). Sources of natural antioxidant compounds are spices, herbs, tea, cocoa, oils, seeds, cereals, grains,

fruits, vegetables, enzymes, proteins and protein hydrolysates (Six, 1994). However, such ingredients can be used only in products when they are compatible with the texture, colour, and flavour of the end product (Rajalakshmi and Narasimhan, 1996). Most useful are antioxidants that are soluble in fats and oils, odourless, tasteless, non-toxic at approved dosage levels and effective in low concentrations (Hauman, 1990). Processing convenience and low cost are also factors. Hence, identification and further purification of the antioxidant compounds become essential for the effective use of natural antioxidants on a commercial basis (Rajalakshmi and Narasimhan, 1996).

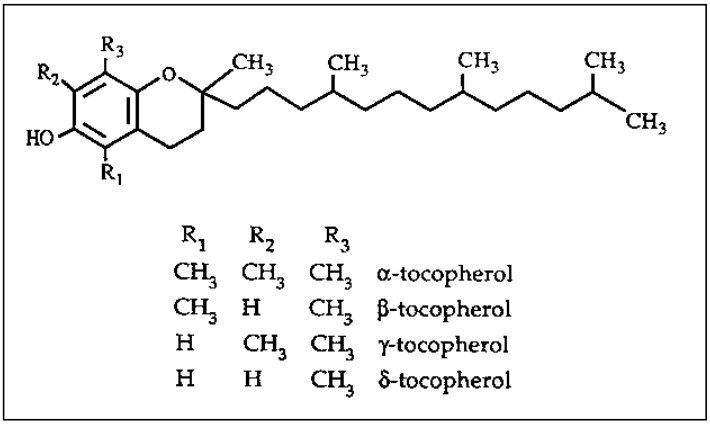
Table 2.3: Natural Antioxidants in some Food Ingredients

Source	Antioxidant
Oils and oilseeds	Tocopherols and tocotriennols: sesamol and related substances, olive oil resins, phospholipids.
Oat and rice brans	Various lignin-derived compounds
Fruits and vegetables	Ascorbic acid: hydroxycarboxylic acids; flavonoids, carotenoids
Spices, herbs, tea and cocoa	Phenolic compounds
Proteins and protein hydrolysates	Amino acids, dihydropyridines, Mailard Reaction Products

2.5.1 Sources of Natural Antioxidants

2.5.1.1 Natural Antioxidants from Vegetable Oils

a. Tocopherols and related compounds



R_1	R_2	R_3	
CH_3	CH_3	CH_3	α-tocopherol
CH_3	H	CH_3	β-tocopherol
H	CH_3	CH_3	γ-tocopherol
H	H	CH_3	δ-tocopherol

Figure 2.9: Tocopherols (Madhavi *et al.*, 1996)

Tocopherols occur as minor constituents in all vegetable oils, and are among the best known and most widely used antioxidants (Pokorny, 1991). The α, β, γ and δ tocopherols (Figure 2.9) differ in the degree of methylation of the dihydrochromanol ring (Pokorny, 1991). Their antioxidant activities range in the order: δ-tocopherol (most effective) > γ-tocopherol > β-tocopherol > α-tocopherol (least effective) (Six, 1994). They work as antioxidants by donating the hydrogen of the hydroxyl group to a fatty peroxide free radical (Six, 1994).

Synthetic DL-α-tocopherol or its acetate derivative is often added to fats and oils, but the addition of natural tocopherols is preferred because the synthetic preparations contain biologically inactive stereoisomers (Pokorny, 1991). Natural tocopherols are readily

available from the sludge formed during the deodorization of refined vegetable oils (Pokorny, 1991). Mixed tocopherols can be formulated to be 100% dispersible in dry ingredients, or can be suspended in water before adding to other materials containing fats and oils, or can be sprayed on using an ethanol carrier (Six, 1994). Usage levels are 0.015-0.045% of mixed tocopherols based on oil or fat content. α-Tocopherol (Vitamin E) is used in high density polyethylene packaging film to stabilize the resin, thus preventing polymer oxidation (Six, 1994).

The tocopherols, however, are more effective in combination with other antioxidants or synergists such as ascorbyl palmitate, ascorbic acid, lecithin, or citric acid than when used alone (Madhavi *et al*., 1996). The antioxidant activity of tocopherol mixtures has been investigated in vegetable oils and animal fats. Individual tocopherols were reported to have prooxidant activities at high concentrations in purified soybean oil (Jung and Min, 1990). In another study, the effectiveness of α- and γ-tocopherols was highly dependent on the test system (bulk oil, emulsion), the concentration, the oxidation time, and the method used to determine lipid oxidation (Huang *et al*., 1995). Huang *et al.* (1995) have since reported that whether tocopherol mixtures acted as antioxidants or prooxidants depended on the concentration of α-tocopherol in the mixtures.

b. Antioxidants from sesame oil and olive oil

The active antioxidant compounds of sesame oil have been found to be quite resistant to oxidation (Pokorny, 1991). The active compounds are produced by lignins in the seeds via various precursors such as sesamolin (Figure 2.10), which is hydrolyzed on heating to form sesamol (Pokorny, 1991). Raw and roasted sesame seed oils are characterized by a higher oxidative stability than other vegetable oils (Rajalakshmi and Narasimhan, 1996). Olive oil is very stable, not only because of its low content of polyenoic fatty acids, but also due to the presence of various natural bitter-tasting antioxidants, mainly derived from hydroxytyrosol (a derivative of pyrocatechol), which is a product of tyrosine degradation, but also formed from various other polyphenols (Pokorny, 1991). During the pressing of olives to make olive oil, the polyphenol-derived antioxidants pass into the raw

oil (Pokorny, 1991). Rape, a major by-product of the mechanical extraction, still has little industrial use and has been found to contain polyphenols, and a method for the extraction and purification from rape was reported by Sheabar and Neeman (1988), using hexane, acetone and ethanol.

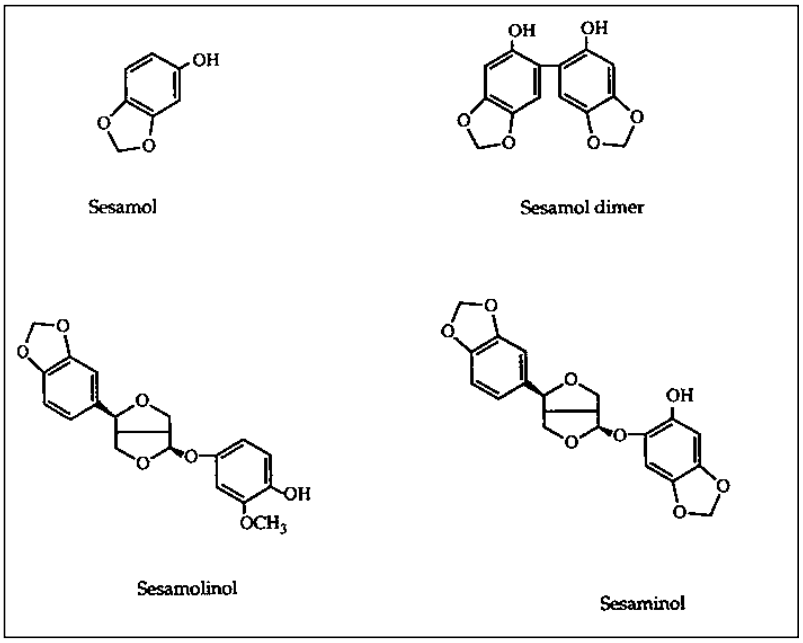


Figure 2.10: Some antioxidant compounds from sesame (Madhavi *et al.*, 1996)

c. Phospholipids

Phospholipids (e.g. lecithin) are present in crude vegetable oils; they are removed during the degumming phase of refining (Pokorny, 1991). Phospholipids (Figure 2.11) are often used as synergists in combination with phenolic antioxidants (Pokorny, 1991). At elevated temperatures some of the phospholipids, particularly phosphatidylethanolomine (PE), greatly enhanced the activity of primary antioxidants in edible oils (Kashima *et al.*, 1991). Phosphadycholine (PC) and phosphatidylserine (PS) are also reported to be

effective (Kashima *et al.*, 1991). The antioxidant activity of phospholipids has been attributed to their ability to decompose hydroperoxides; the antioxidant activity appears to be dependent on the presence of bound choline or ethanolamine as a constituent of the phospholipids (Pokorny, 1991). The other possible mode of action in phospholipids is through the release of protons and decomposition of hydroperoxides without the formation of free radicals or via the regeneration of primary antioxidants (Jadhav *et al.*, 1996).

Kashima *et al.* (1991) have reported that the oxidative stability of perilla oil was especially increased by addition of PE and PS. The addition of phospholipids and α-tocopherol to buffalo butterfat increased its oxidative stability (Six, 1994). When these two antioxidants were used in various concentrations, the protection factor was strongly enhanced at higher levels of phospholipids.

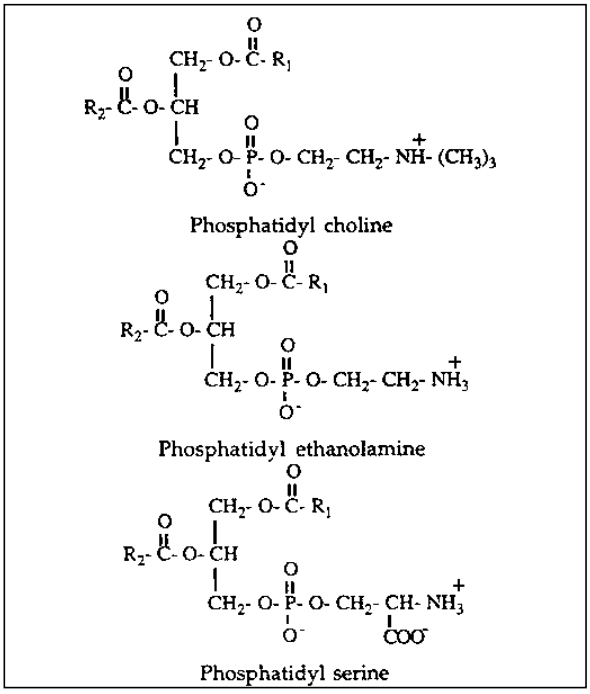


Figure 2.11: Major phosphatides in soybean lecithin. R_1, R_2 are fatty acid residues;

R_3 represents inositol.

2.5.1.2 Natural Antioxidants from Cereals

Cereals are amongst the most common food components, and can be added to many food products. The antioxidants from cereals originate from lignins, which are present in many plants (Pokorny, 1991). Their metal-scavenging abilities contribute to their stabilizing effects (Pokorny, 1991). Oat flour and oat extracts were among the first antioxidants proposed for use in the stabilization of fats, oils and fat-containing foods (Pokorny, 1991). Esters of caffeic and ferulic acids appear to be the most important antioxidant in oats (Pokorny, 1991). Similar compounds, such as oryzanol (an ester of ferulic acid and triterpenic alcohol), are found in the brans of other cereals (Pokorny, 1991). Some sterols in oats have been shown to retard thermal changes at frying temperature (Duve and White, 1991). These sterols have a side chain containing an ethylidiene group on the sterols that isomerize to produce an allylic free radical, which can interrupt the oxidation chain (Duve and White, 1991). Thus, the addition of sterols may be a natural alternative to the use of antioxidants in frying oils. In a study determining the effect of different cereal-water suspensions on the oxidation of linoleic acid, oat suspension was the best antioxidant amongst wheat, barley and rye (Lehtinen and Laakso, 1997).

2.5.1.3 Natural Antioxidants from other Sources

a. Phenolic compounds

Various phenolic compounds, such as hydroxyl derivatives of cinnamic and benzoic acids, are common constituents of many plant foods; most possess antioxidant activity (Pokorny, 1991). The bioactivity of phenolics may be related to their antioxidant behavior, which is attributed to their ability to chelate metals, inhibit lipoxygenase, and scavenge free radicals (Amorowicz *et al.*, 2004; Decker, 1997). Since phenolics are found in virtually all plant foods and often at very high concentrations (Table 2.4), these compounds are consumed in quantities of up to 1 g per day (Decker, 1995). When tested

in edible oils, and in fish, meat and poultry products, phenolic rich extracts haves shown antioxidant activities comparable to that of synthetic antioxidants (Balasundram *et al.*, 2006). The presence of conjugated ring structures and hydroxyl groups allows phenolics to actively scavenge and stabilize free radicals (Decker, 1995). The presence of carboxylic acid groups in numerous phenolic compounds can result in the inhibition of lipid oxidation by metal chelation (Decker, 1995). Dihydroxy derivatives are more efficient that monohydroxy derivatives; the derivatives of cinnamic acid are more effective than those of benzoic acid; and the presence of one or two methoxy groups in the ortho position to the hydroxyl group increases the antioxidant effect (Marinova and Yanishlieva, 1994). Esters of caffeic acid (e.g. chlorogenic acids) are the most important plant-derived phenolic antioxidants, as they have high antioxidant activity at levels found in natural substances (Pokorny, 1991). Phenolic compounds have also been isolated from potato peel waste, and they mainly consist of chlorogenic, gallic, protocatechuic and caffeic acids (De Sotillo *et al.*, 1994).

Table 2.4: Examples of Phenolic Compounds in Foods (Source: Decker, 1995)

Compound	Occurrence
Vanillin	Vanilla beans, cloves
Sesamol	Sesame seeds
Caffeic acid	Oats, soybeans, blueberries, prunes, grapes
Quercetin	Tea, coffee, cereal grains, onions
Epicatechin	Tea leaves
Epigallocatechin	Tea leaves
Ellagic acid	Grapes, strawberries, raspberries
Curcumin	Turmeric, mustard

Cieslic *et al.* (2006) reported broccoli in having the highest content of polyphenols when analyzing various fruits and vegetables. Gum guaiac is a naturally occurring phenolic antioxidant from the wood of *Guajacum officinale* L. or *G. sanctum* L. It was widely used in the 1940s as an antioxidant for oils and fats, especially for the stabilization of lard

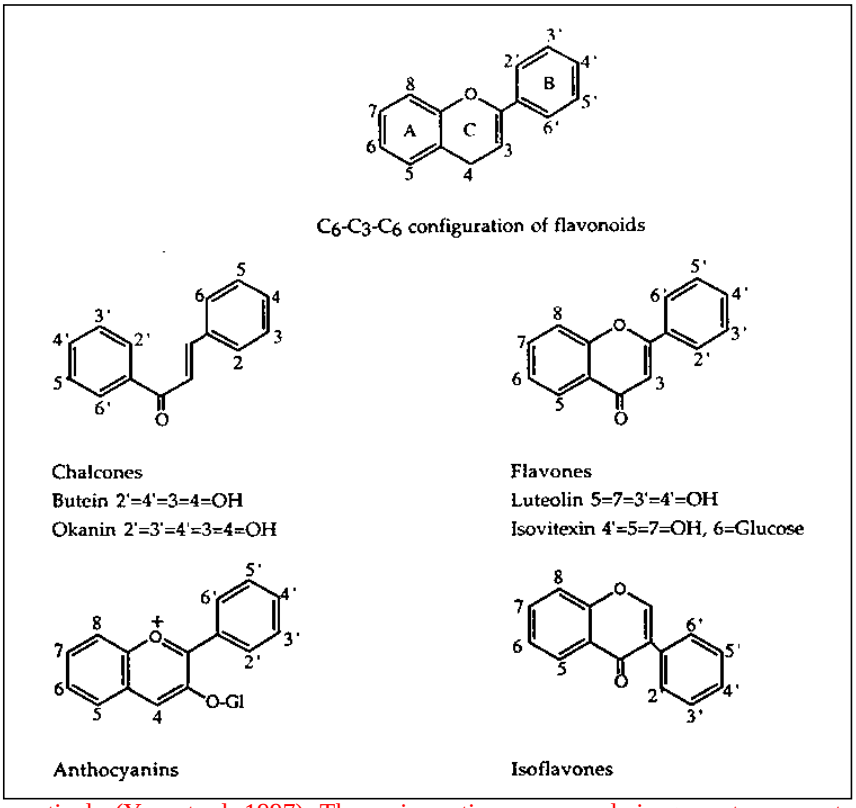
(Rajalakshmi and Narasimhan, 1996). Noordihydroguaiaretic acid is a phenolic natural antioxidant obtained from the creosote bush (*Larrea divaricata*) that was widely used in food products in the 1950s and 1960s (Rajalakshmi and Narasimhan, 1996). At least nine phenolic acids, including syringic, vanillic, caffeic, ferulic, p-coumaric and p-hydroxybenzoic acids have been identified and isolated from soybeans and defatted soybean flour (Pratt and Birac, 1979).

Phenolic compounds exhibit anticarcinogenic activity in numerous animal models. The antioxidant activity of phenolics is thought to be involved in their anticarcinogenic properties (Decker, 1995). Kato *et al.* (1997) reported that some phenolic antioxidants (caffeic acid, curcumin, and cinnamic acid) show strong inhibitor effects on nitration of tyrosine residue by peroxynnitrite, thus preventing modification of collagen.

b. Flavonoids

Flavones and related compounds occur widely in many plant foods. The chemical structures are based on C_6-C_3-C_6 (Rajalakshmi and Narasimhan, 1996). Flavonoids function as primary antioxidants, chelators and superoxide anion scavengers (Rajalakshmi and Narasimhan, 1996). The presence of hydroxyl groups at the 3'-, 4'-, and 5'-positions enhances antioxidant activity compared to that of a single hydroxyl group (Rajalakshmi and Narasimhan, 1996). The major subgroups are flavonols-flavones, isoflavones, catechins, proanthocyanins, and anthocyanins – Figure 2.12 (Rajalakshmi and Narasimhan, 1996). Chalcones, flavonones, leucoanthocyanins, and dihydroflavonols are the common precursors for the different subgroups in the biosynthetic pathway (Rajalakshmi and Narasimhan, 1996). The antioxidant ability of flavonols (3-hydroxyflavone derivatives) has been well established in both non-aqueous lipid systems and in aqueous systems (Pratt and Watts, 1963). Cinnamic and phenolic acids are closely related to flavonoids, and some of them are precursors for the flavonoid biosynthetic pathway (Rajalakshmi and Narasimhan, 1996).

Catechin and quercetin (Figure 2.13) , flavonoid pyrocatechol derivatives, possess high antioxidant activity and have been used to stabilize lard (Pokorny 1991). Some flavonoid pyragallol derivatives, such as tea tannins, also possess antioxidant activity (Pokorny 1991). Polyphenols are the most abundant group of compounds in fresh tea leaves and are found in green and black tea beverages at 30-42% and 3-10% of the total dry matter,



respectively (Yen et. al. 1997). The major active compounds in green tea are catechins with the following order of activities: epigallocatechin gallate > epigallocatechin > epicatechin gallate > epicatechin (Rajalakshmi and Narasimhan 1996). In a previous study, Yen and Chen (1995) investigated the relationship between antioxidant activity and the antimutagenecity of various tea extracts (green tea, pouchong tea, oolong tea, and black tea). All tea extracts exhibited markedly antioxidant activity and reducing power, especially oolong tea, which inhibited 73.6% peroxidation of linoleic acid. Tea flavonols were compared in a dose-response manner with vitamins C and E and beta-

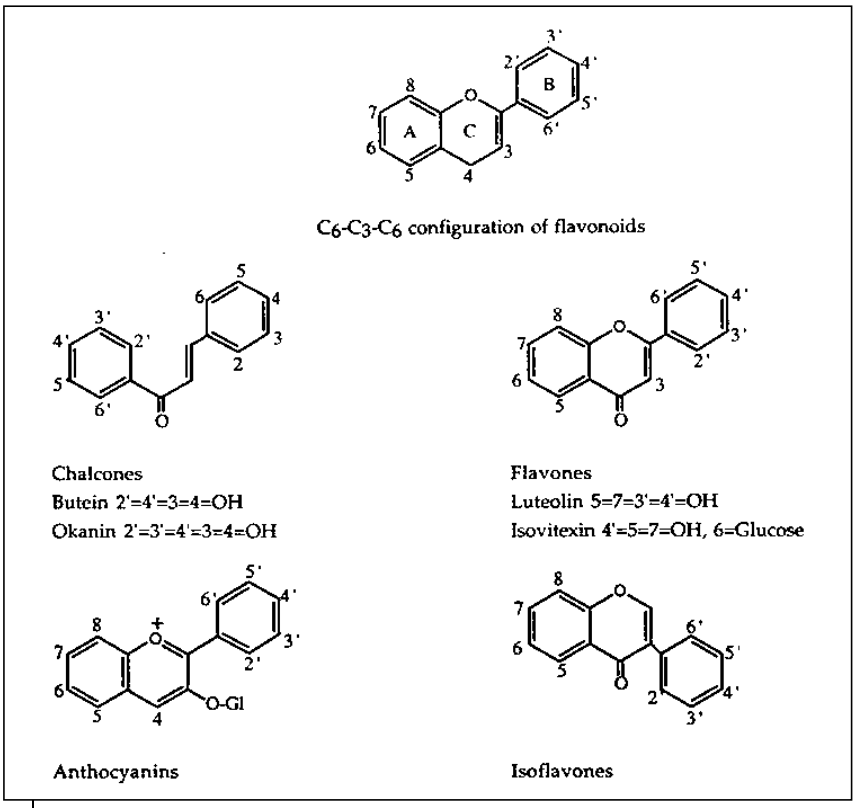


Figure 2.12: Some antioxidant flavonoids and related compounds (Madhavi *et al.*, 1996)

Catechin and quercetin (Figure 2.13) , flavonoid pyrocatechol derivatives, possess high antioxidant activity and have been used to stabilize lard (Pokorny, 1991). Some flavonoid pyragallol derivatives, such as tea tannins, also possess antioxidant activity (Pokorny, 1991). Polyphenols are the most abundant group of compounds in fresh tea leaves and are found in green and black tea beverages at 30-42% and 3-10% of the total dry matter, respectively (Yen and Chen., 1997). The major active compounds in green tea are catechins with the following order of activities: epigallocatechin gallate > epigallocatechin > epicatechin gallate > epicatechin (Rajalakshmi and Narasimhan,

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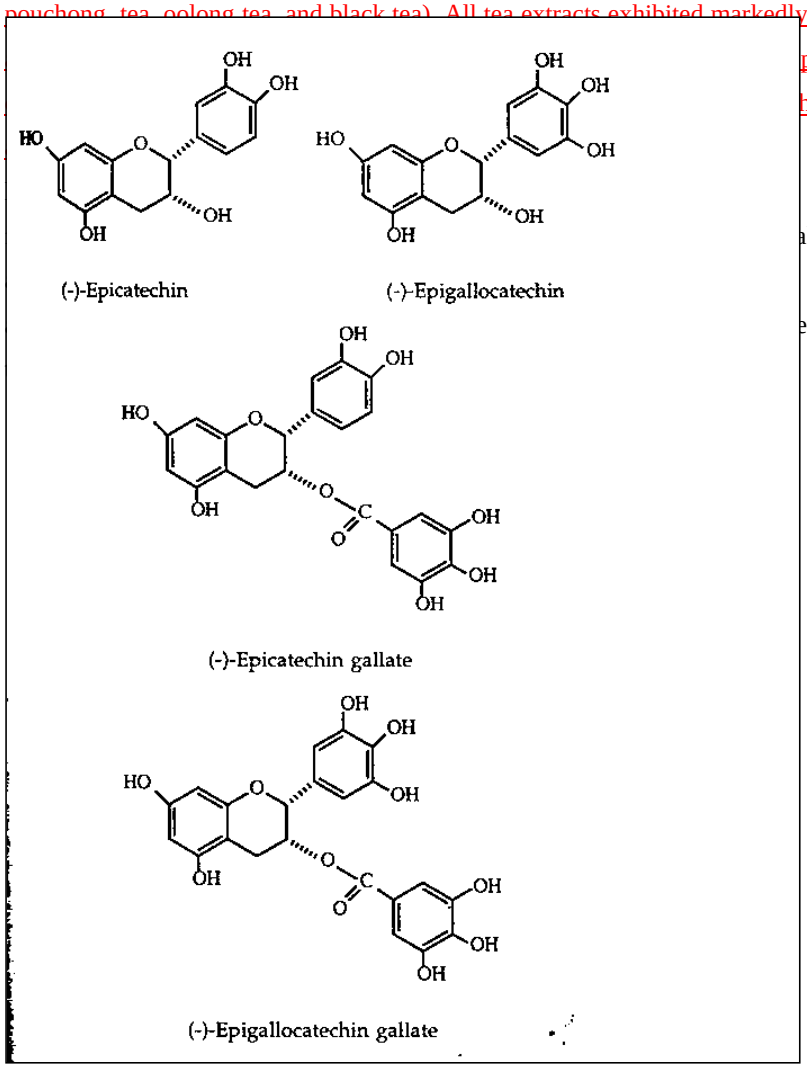

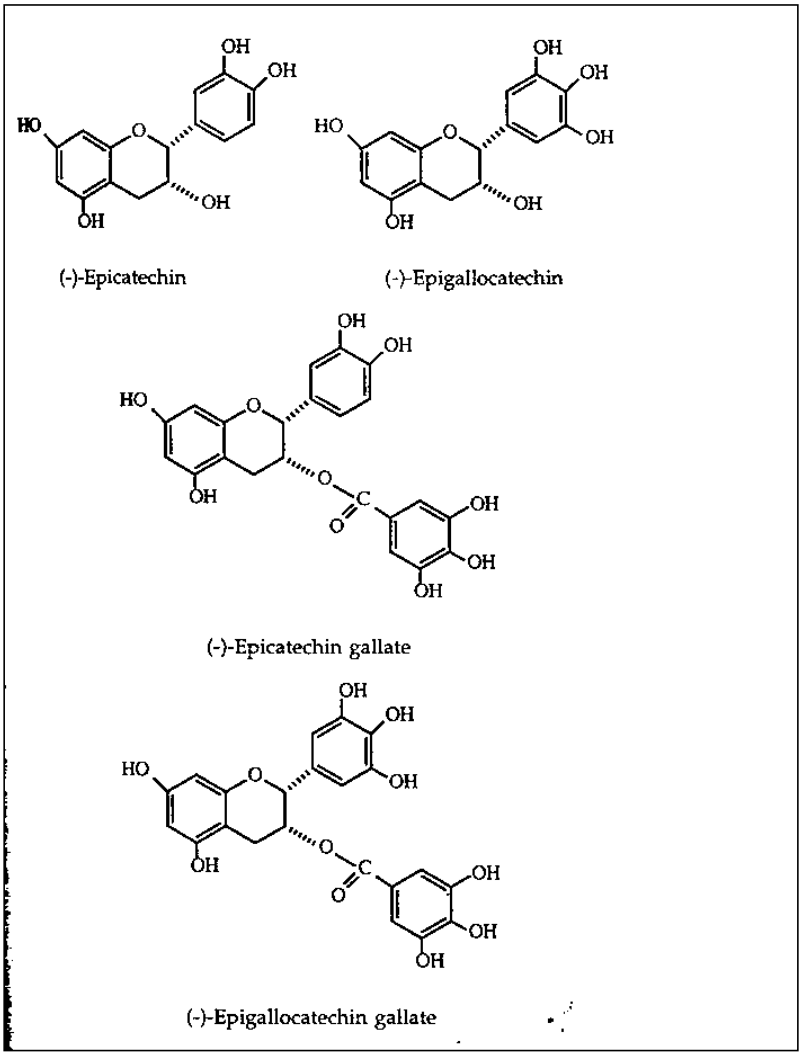

Figure 2.13: Some antioxidant compounds from green tea (Madhavi *et al.*, 1996)

Vekrari *et al*. (1993) reported that the major antioxidant factors isolated from oregano consisted of flavonoids. Subsequent chromatographic and spectrophotometric analysis demonstrated the presence of the flavone apigenin, the flavonone, eriodictyol and the dihydroflavonols, dihydrokaempferol and dihydroquercetin. Methanol extracts of cottonseeds were found to possess antioxidant components, with subsequent

chromatographic techniques indicating that the flavonoids quercetin and rutin were the major flavonoids present (Whittern *et al.*, 1984). *Vernonia amygdalina*, a traditional herbal medicine used as an antimalarial and a laxative herb in Nigeria, was found to contain three flavones: luteolin, luteolin 7-O-β-gluconosidae and luteolin 7-O-β-glucoside (Igile *et al.*, 1994). Hot methanol extracts of Spanish peanuts were found to possess antioxidant activity and the major antioxidant component was identified as a flavonoid-dihydroquercetin (Pratt and Miller, 1984). Chia seeds were also found to be a source of flavonoid antioxidants (Taga *et al.*, 1984).

c. Antioxidants from botanicals, herbs and spices

Dietary antioxidants such as water soluble vitamin C, and phenolic compounds, as well as lipid soluble vitamin E and carotenoids, present in vegetables contribute to the first and second defense lines against oxidative stress (Podsedek, 2006). Brassica vegetables belong to the Cruciferous family, and include different genus of cabbage (white, red, savoy, swamp, Chinese), cauliflower, broccoli, brusselsprouts and kale. These vegetables possess both antioxidant and anticarcinogenic properties (Cohen *et al.*, 2000; Chu *et al.*, 2002).

The antioxidant activities of various spice extracts have long been recognized from practical experience (Pokorny, 1991). For example, garlic and red chilli were added to butterfat (ghee) and red chilli, fennel or cloves were often used in the preparation of pickles (Madsen and Bertelsen, 1995; Rajalakshmi and Narasimhan, 1996). Several compounds have been identified - Figure 2.14.

Early investigations have demonstrated that rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) are two of the most potent spices as natural antioxidants, among spices prompting a great deal of experimental work aimed at isolating and identifying the compounds responsible for the antioxidant activity (Rajalakshmi and Narasimhan, 1996). High content of phenolic compounds was established in various *Salvia* species (Millauskas *et al.*, ~~, Venskutonis, van Beek~~ 2004). Carnosol (an odourless

and tasteless phenolic diterpenic lactone) and carnosic acid have been found in rosemary (Madsen and Bertelson, 1995; Pokorny, 1991). The same compounds have been isolated from sage, indicating a close botanical relationship between the two species (Madsen and Bertelsen, 1995).

Rosmanol, epirosmanol, isorosmanol and rosmarinic acid have all been isolated from rosemary (Madsen and Bertelsen, 1995). A standardized oleoresin of rosemary extract has many different phenolic components (Six, 1994). It is thought they act in synergy to provide antioxidant activity. Furthermore, the two diterpenes rosmaridiphenol and rosmariquinone have been found in rosemary (Madsen and Bertelsen, 1995). Data also indicates that the most effective antioxidant components from sage and rosemary were carnosol, rosmaric acid, and carnosic acid, followed by caffeic acid, rosmanol, rosmadial, genkwanin and cirsimantin (Cuvelier *et al*., 1996). Chang and co-workers (1977) reported on a patented process for the extraction of rosemary and sage followed by a vacuum distillation of the extract in an edible oil or fat to obtain a bland natural antioxidant. Djarmati and colleagues (1991) separated the ethanol extract of sage into 5 fractions through extraction with supercritical fluid.

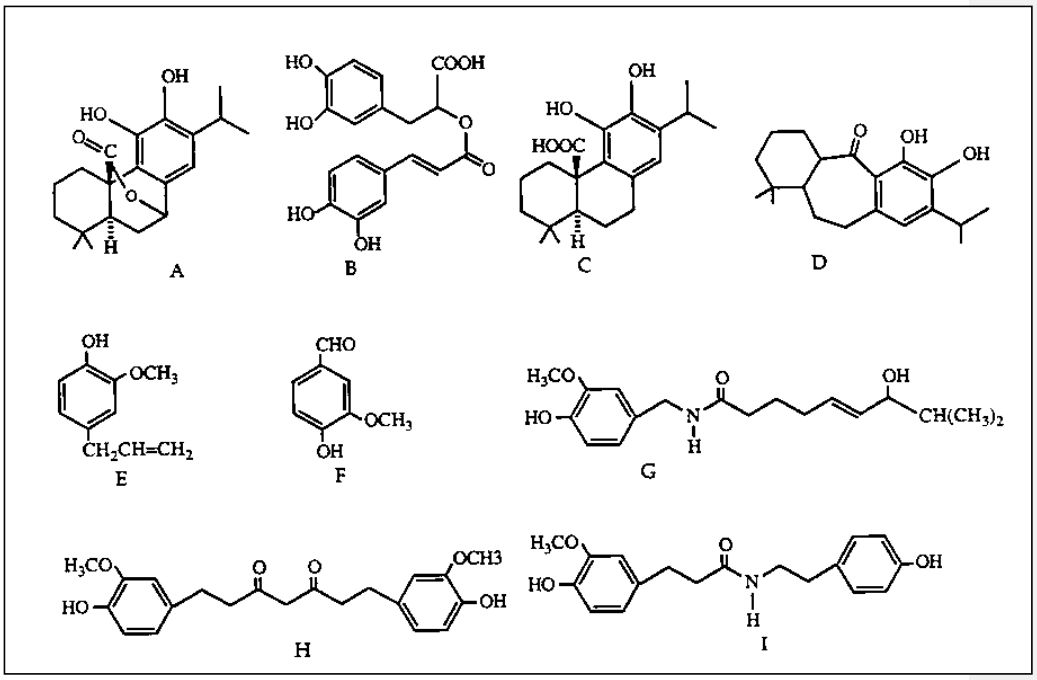

Figure 2.14: Some antioxidant compounds from spices. (A) Carnosol from rosemary; (B) rosmarinic acid from rosemary; (C) carnosic acid from rosemary; (D) rosmaridiphenol from rosemary; (E) eugenol from cloves; (F) vanillin from vanilla pods; (G) capsaicin from capsicum; Htetrahydrocurcumin from turmeric; (I) ferulic acid amide from black pepper.) (Madhavi *et al.*, 1996)

Rosmaridphenol, the diphenolic diterpene from rosemary when tested in lard; its antioxidant activity of this compound was superior to BHA (Houlihah *et al.*, 1984). A natural antioxidant with activity greater than BHA and equal to BHT in rosemary leaves was identified as carnosol (Wu *et al.*, 1982). Oleoresin rosemary extract and sage extract retarded oil deterioration during deep fat frying of potato chips in palmolein (Jaswir *et al.*, 2000). Rosemary extracts have achieved widespread industrial application. Crude extracts are of green colour and have a rather strong rosemary odour (Loliger and Wille, 1993). Therefore their application is restricted to foods where these properties do not

present a disadvantage. Areas of applications of these antioxidants are for instance cereal flakes and meat products like pork patties (Loliger and Wille, 1993).

~~Five different phenolic compounds have been isolated from the methanol extract of oregano (*Origanum vulgare* L.); all five showed antioxidant activity (Madsen and Bertelsen 1995). The five antioxidant phenolic acids are: phenyl glucoside, protacechuic acid, caffeic acid, rosmarinic acid and a new compound established as 2-caffeoyloxy 3-{2 (4 hydroxybenzyl) 4,5 dihydroxy} phenylpropionic acid (Six 1994). One of the compounds was identified as rosmarinic acid (Madsen and Bertelsen 1995). In addition, carvacrol and thymol have been isolated from the essential oil of oregano (Madsen and Bertelsen 1995). Oregano showed antioxidant activity in stabilizing lard against oxidation (Vekiari et. al. 1993).~~

Eugenol and gallic acid were found to contribute to the antioxidant activity of clove (*Eugenia caryophyllata* Thumb.) (Madsen and Bertelsen, 1995; Rajalakshmi and Narasimhan, 1996). In turmeric (*Curcuma longa* L.), the water-soluble peptide turmerin and the lipid soluble curcumin have been isolated, and both have demonstrated antioxidant activity (Madsen and Bertelsen, 1995). Curcumin was also found to be present in ginger (*Zingiber officinalis* Roscoe.) (Madsen and Bertelsen, 1995). Twelve compounds isolated from ginger exhibited higher activity than alpha-tocopherol (Kikuzaki and Nakatani, 1993).

Thymol and its biphenol derivatives have been reported to be active in thyme (Rajalakshmi and Narasimhan, 1996).The antioxidative activity of mint species is due, in part, to rosmarinic acid, flavonoids and other derivatives of hydroxycinnamic acid (Duke, 1992). Summer savory, *Satureja hortensis* L., a culinary herb widely used in the food industry has been found to contain the antioxidant compound rosmarinic acid (Bertelsen *et al*., 1995). Antioxidant substances in red pepper have been identified as alpha-tocopherol and capsaicins (Fugimoto *et al*., 1974). In capsicum, in addition to capsaicin and dihydocapsaican, a new tasteless compound with antioxidant properties has been isolated (Rajalakshmi and Narasimhan, 1996). Vanillin, used extensively as a food

flavouring agent, has potent antioxidant properties even in dry mixes like rice flakes (Six, 1994; Rajalakshmi and Narasimhan, 1996).

Farag *et al.* (1989) tested the effectiveness of various essential oils on linoleic acid oxidation, and ranked them accordingly: caraway > sage > cumin > rosemary > thyme > clove. They concluded there was a relationship between the antioxidant effect and the chemical composition of the oils. Some commercially available herb extracts were assessed in preserving alpha-tocopherol in sunflower oil during heating at 85-105^{0}C (Beddows *et al*., 2000). Rosemary, thyme, turmeric, sage, oregano and cumin extracts delayed rancidity. Some preservation was observed with clove extract but coriander and ~~cardamon~~cardamom extracts were pro-oxidants. The antioxidant activities of methanol extracts of oregano, dittany, thyme, marjoram, spearmint, lavender and basil were tested in lard stored at 75^{0}C (Economou *et al*., 1991). Clove and thyme oils at various concentrates exhibited antioxidant activity in preventing the oxidation of cottonseed oil, with clove oil proved superior to that of thyme oil. Garlic has been shown to protect against oxidation by t-butylhydroperoxide (O'Brien, 1990).

2.6 ANTIOXIDANT VITAMINS

Antioxidant defense to oxidative stress is achieved both by enzymatic reactions (e.g. compartmentalized superoxide dismutase, catalase and glutathione peroxidase and glutathione reductase tissue enzyme systems) and by non-enzymatic reactions (e.g. vitamins and micronutrients) (Kitts, 1997). Lipid oxidation, both enzymatic and non-enzymatic is an important factor in the deterioration of food and cosmetic products as well as having health implications. Antioxidants reduce free-radical lipid oxidation by acting as either hydrogen donors or free-radical scavengers; the mechanisms that are involved include decomposition of peroxides, singlet oxygen inhibition and free-radical acceptors, as well as metal-chelating activity (Kitts, 1997). There are however, certain situations where specific dietary antioxidants can carry out these functions. Antioxidant vitamins, reported to curb damaging oxidative reactions in the body, are increasingly viewed as a significant factor in reducing the risk of regenerative diseases such as cancer

and cardiovascular disease (Ranhotra *et al*., 1995). The main antioxidant vitamins are vitamin C, vitamin E, and beta-carotene. Although beta-carotene is not strictly a vitamin, it is commonly included in the 'antioxidant vitamin group', as it is a vitamin A precursor.

2.6.1 Vitamin E

Vitamin E refers to a group of eight naturally occurring compounds - alpha, beta, gamma and delta tocopherols (Landvik *et al.*, 1996). Vitamin E is the major chain-breaking antioxidant in body tissues as is considered, the first line of defense against lipid peroxidation, protecting cell membranes at an early stage of free radical attack (Landvik *et al*., 1996). The biologically and chemically most active form of vitamin E is alpha-tocopherol (Frei, 1994). Alpha-tocopherol is a lipid-soluble compound present in biological membranes and lipoproteins; it is by far the most abundant lipid soluble antioxidant in humans (Frei, 1994).

$LO_2^{\cdot} + LH \longrightarrow LOOH + L^{\cdot}$, where LH, $L^{\cdot}$, $LO_2^{\cdot}$ and LOOH are lipid, lipid radical (carbon centered), lipid peroxy radical, and lipid hydroperoxide respectively.

$LO_2^{\cdot} + TOH \longrightarrow LOOH + TO^{\cdot}$, where TOH and $TO^{\cdot}$ are alpha-tocopherol and alpha-tocopheroxyl radical respectively.

The hydrophobic character of alpha-tocopherol enables it to be a strong antioxidant in lipid systems, where it functions as a radical scavenger and terminates the propagation of radical chain reactions by reacting with peroxyl radicals and generating unreactive phenoxyl radicals and hydroperoxide products (Kitts, 1997).

It is known that alpha-tocopherol scavenges singlet oxygen as well as oxygen radicals (Niki, 1996). It is accepted that the major function of vitamin E *in vivo* is an antioxidant

that protects cells and tissues from oxidative damage induced by free radicals (Niki, 1996). Vitamin E interrupts the chain of membrane lipid peroxidation and is thus a "chain-breaking" antioxidant (Packer, 1994). It may also stimulate immune response, inhibit cancer initiation lessen the severity of prostaglandin-mediated disorders, and inhibit the conversion of nitrites to nitrosamines, which are strong tumour promoters (Niki, 1996). It is lipophilic and located in the lipophilic compartment of membranes and lipoproteins (Niki, 1996). Dietary supplementation with vitamin E increases the plasma tocopherol concentration and the potential for associated antioxidative protection (Kitts , 1997).

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2.6.2 Vitamin C

Ascorbic acid is a water-soluble antioxidant with significant interspecies and intraspecies variations in its metabolism Ascorbic acid (vitamin C) is a commonly used antioxidant in many food systems for maintaining organoleptic quality (Kitts, 1997). In its natural forms, ascorbic acid [e.g. L-ascorbic acid and D-isoascorbic acid (erythorbic acid)] functions as a reducing agent (Figure 2.15) and, as an oxygen scavenger (e.g. 3.5 mg of ascorbic acid will scavenge the oxygen contained in a 1 cm^3 headspace) (Kitts 1997). In addition, the metal-sequestering activity of ascorbic acid, which forms metal-ascorbate complexes that are less reactive with oxygen than with metal ions alone, provides antioxidant activity (Kitts, 1997). The oxygen scavenging activity of ascorbic acid is effective in trapping both singlet oxygen and superoxide anion, thus producing an ascorbate free radical (Kitts, 1997). Ascorbate radicals so produced also react with peroxyl radicals to produce hydroperoxides (non-radical species) and the oxidized form of ascorbic acid, namely dehydroascorbic acid (Kitts, 1997). The conversion of native ascorbic acid to a salt form increases its stability and versatility in different food systems at the expense of biological activity [e.g. ascorbic acid (100%), calcium ascorbate (53%) and ascorbyl palmitate (42%)]. (Kitts, 1997).

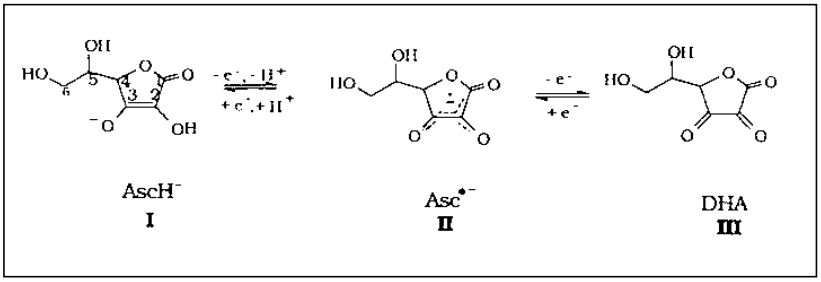

Figure 2.15: Reduction of Ascorbic acid (Cadenas & Packer, 1996)

In muscle systems, ascorbic acid has been used to delay the formation of metmyoglobin in fresh meat products, it has also been used to prevent enzymatic browning in fresh fruit and vegetables (Kitts, 1997). The addition of ascorbates and sodium citrate to milk also provides protection against the loss of the lipid-soluble vitamins A and D (Kitts, 1997). Unlike vitamin E, ascorbic acid activity occurs in the cytosolic compartments of the cell, because it is water soluble (Kitts, 1997). Vitamin C, located in the blood plasma, is assumed to be able to regenerate oxidized vitamin E located in the LDL, thereby permitting the continued inhibition of lipid peroxidation (Germann, 1990):

$$TO^{\cdot} + AsCH^{-} \longrightarrow TOH + AsC^{\cdot -}$$

Ascorbate is also used as a pro-oxidant. This paradoxical behavior results because it is an excellent reducing agent (Buettner and Jurkiewic, 1996). As a reducing agent it is able to reduce catalytic metals such as Fe^{3+} and Cu^{2+} to Fe^{+} and Cu^{+} . In general, low concentrations of ascorbate are required for pro-oxidant conditions, while high concentrates are needed for antioxidant conditions (Buettner and Jurkiewic, 1996).

2.6.3 Beta-carotene

In recent years, there has been an expansion of interest in the ability of carotenoids to scavenge different forms of active oxygen. Carotenoids have been shown to be excellent scavengers for triple excited states and for singlet oxygen, and this indicates an

important role for carotenoids as antioxidants in photosynthesis and as protective compounds for certain human photosensitivity disorders (Handelman, 1996). The lengthy experimental history of plant biochemistry documents the ability of carotenoids to absorb energy form molecules in high-energy states, a process referred to as the quenching of excited states (Handelman, 1996). Since 1980, a substantial body of evidence has developed that carotenoids can scavenge peroxyl radicals and related forms of active oxygen, in addition to their well-defined ability to quench excited-state triplets and singlet oxygen (Handelman, 1996).

The prototypical carotenoid is beta-carotene. The term carotenoid generally designates a long-chain molecule with 40 carbon atoms and an extensive conjugated system of double bonds (Handelman, 1996). Like alpha-tocopherol, beta-carotene is a lipid-soluble substance (Frei, 1994). In addition to acting as a Vitamin A precursor, beta-carotene is a very effective quencher of singlet oxygen and can also inhibit lipid peroxidation (Frei, 1994). It has been shown that one molecule of beta-carotene can quench at least 1000 molecules of singlet oxygen without itself undergoing destruction (Handelman, 1996). Interestingly, beta-carotene exhibits effective radical-trapping antioxidant behavior only at low (physiologic) oxygen pressures (Frei, 1994). The reactivity of beta-carotene towards peroxy radicals and the stability of the resulting carbon-centered radicals are two important features that give the carotene molecule antioxidant capabilities (Basu and Dickerson, 1996). Beta-carotene may be able to complement vitamin E substantially in the lipid phase since beta-carotene in contrast to the latter is most protective at low partial pressures of oxygen (<20%) whereas vitamin E is particularly active at high oxygen pressures (>20%) (Germann, 1990).

The proposed mechanism of antioxidant activity for beta-carotene involves its hydrophobic character and propensity to quench singlet molecular oxygen (Kitts, 1997). This vitamin inactivates triplet photochemical sensitizers (e.g. plant chlorophyll a; and hematoporpyhrin and myoglobin in animal tissue), thus interfering with lipid peroxidation by trapping chain propagating lipid peroxyl radicals (Kitts, 1997). Green plants would be unable to survive without single oxygen quenching carotenoids

(Charleux, 1991). The reactivity of beta-carotene towards peroxyl radicals and the stability of the resulting carbon-centered radical; are two important features that give the molecule antioxidant capability (Burton, 1989).

2.7 APPLICATIONS OF ANTIOXIDANTS

2.7.1 Food Industry

Shelf-life is a major consideration in developing, and marketing many food products. Consumers desire products that maintain fresh appearance, odour, and flavour for long as possible, and longer shelf-life reduces costs for the producer and, ultimately, for the consumer (Byrd, 2001). Moreover, the storage capacity of food is becoming more and more important due to the increasing amount of manufactured foods needed for feeding the growing world population and due to the demographic shift from rural to urban areas (Loliger and Wille, 1993). A steady demand for longer shelf life is especially required in the industrialized countries where there are prolonged times between raw material production and food consumption (Loliger and Wille, 1993).

The shelf-life of foods is often limited. Their stability is in general restricted due to reactions such as oxidative degradation of solids, browning (Maillard reaction) and degradation of starch (Loliger and Wille, 1993). In connection with these processes, unpleasant tastes are often observed. Such off-flavours may be described as for instance as rancid, loss of flavour, stale, etc. During autoxidation, a number of compounds may be produced from the initially formed hydroperoxides (Burkow *et al*., 1995). Many of these are low-molecular weight compounds with strong odour and taste, which cause the characteristic sensory attributes of rancid lipids (Burkow *et al*., 1995). These problems concern nearly all foods composed of the three main food components lipids, carbohydrates and proteins (Loliger and Wille, 1993). In addition to reducing the quality and nutritional value of foods, lipid oxidation products, such as peroxides, aldehydes, and ketones are harmful to human health (Byrd, 2001).

Lipid oxidation leading to rancidity is often the decisive factor for microbial spoliage determining the useful storage life of food products, even when the fat content is very low (Frankel, 1993). Protection of foods against lipid oxidation usually involves exclusion of oxygen by packing in vacuum or inert gases and/or the addition of antioxidants (Alaiz *et al*., 1995). Use of antioxidants is thus a means of reducing the rancidity of fats and oils in foodstuffs. Many types of compounds have been used as food antioxidants over the years, including those that function as free radical scavengers, oxygen absorbers, and chelators (Nunez-Delicado *et al*., 1997). Food antioxidants have been used commercially for the last 50 years but their general use probably dates back to early man, who first used fire and smoke, and later spices and flavourings, to preserve foods (Haumann, 1990). In addition, antioxidants represent one of the oldest established groups of modern food additives (Coppen, 1990). Food manufacturers continue to add antioxidants during food processing to minimize lipid oxidation (Aruoma, 1996). Antioxidants should be seen, as one of several measures available but used properly they are generally effective, easily applied and inexpensive (Frankel, 1993). Most useful are antioxidants that are soluble if fats and oils, odourless, tasteless, nontoxic at approved dosage levels and effective in low concentrations (Loliger and Wille, 1993).

The addition of antioxidants to fats, oils and foods containing fats and oils is desirable for several reasons. Antioxidants can increase the shelf-life of foods by 15-200%, allowing food to be transported and stored for longer periods (Duve and White, 1991). Antioxidants are used to protect fats and oils from developing rancid flavours and odours from decomposition during storage (Haumann, 1990). Antioxidants also minimize the oxidative destruction of certain vitamins and essential amino acids, and prevent the spoilage of many foods containing fats or oils (Haumann, 1990). Besides their main objective of extending the shelf-life of foods, chain breaking and secondary antioxidants can also widen the range of oils and fats which can be used for certain products (Loliger and Wille, 1993). Antioxidants can be effectively employed in a wide range of food products that are relatively easy to incorporate and have a broad range of activities that can withstand the various food processing operations (Madhavi *et al*., 1996). One of the

major advantages of this is that the food manufacturer will be in a position to select the best possible antioxidant from a wide array of compounds or design new combinations and concentrations to suit the needs of specific food products (Madhavi *et al*., 1996). In addition, the naturally occurring antioxidant systems are impaired during processing, making processed food more susceptible to oxidation (Madsen and Bertelsen, 1995). The addition of antioxidants to these products will compensate for the loss of the natural antioxidants. Antioxidants are used in shortenings, cooking oils, potato chips, breakfast cereals, salted nuts and other foods, and are added to the packaging material for some products (Haumann, 1990).

Foods that may require stabilization include most edible oils, lard, fats and oils used for frying, fats and oils used in processed foods, margarines that contain animal fats, milk fat' fried foods such as potato chips, roasted nuts, dried soups, broths and seasonings, dried meat, frozen fish and fish oil, dried milk, potato powder, flake and granules, cakes, chewing gum, concentrated vitamin preparations, flavourings and essential oils (Pokorny, 1991). Effective stabilization of oils and shortenings extends the shelf life of end products in which they are used (Haumann, 1990). Antioxidants cannot reverse oxidation nor regenerate a rancid product. Thus, it is crucial to add an antioxidant to a freshly produced fat in oil before the oxidation process has begun (Haumann, 1990). The most widely used antioxidants are still the traditional synthetic products, with BHA, BHT, TBHQ and PG dominating markets around the world (Haumann, 1990). BHT is very effective in vegetable oils and may be lost during frying because of its steam volatility (Gordon, 1996). BHA has good stability and is an effective antioxidant in fried foods, but it contributes little to increasing the stability of oils containing tocopherols (Gordon, 1996). Propyl gallate (PG) provides better oxidative stability to lard and rendered poultry fat than BHA and BHT (Madhavi *et al*., 1996). The potency of TBHQ is equivalent to or greater than BHA, BHT, or PG in stabilizing a variety of crude and refined vegetable oils (Madhavi *et al*., 1996).

2.7.2 Disease Prevention

Growing evidence of the role of free radicals and antioxidants in health and ageing has focused public interest on these compounds - even if not everybody is clear what they are (Aruoma, 1996a). During the past decade it has become increasingly evident that oxygen free radical damage to cellular constituents is implicated in a number of pathological processes, including carcinogenesis, arthritis, adult respiratory-distress syndrome, emphysema, retinopathy of premature aging, atheroclerosis, muscular dystrophy, and ischemia-reperfusion tissue injury (Stadtman, 1991). Each cell in the body is exposed to numerous free radical attacks, each capable of forming damaging peroxides and hydroxyl radicals. Unless controlled, these reactive agents can quickly cause extensive damage to tissue and induce inflammation, disease and/or death (Kinsella *et al*., 1993).

Free radicals and active oxygen species can attack lipids, proteins, sugars, carbohydrates, and DNA to induce oxidation, cleavage, cross linking, and modification, which eventually cause damage (Niki, 1991). Humans, being oxygen-dependent organisms, are always at risk of free-radical damage (Deshpande *et al*., 1996). The body naturally produces free radicals as it metabolizes oxygen (Deshpande *et al*., 1996). Free radicals and reactive oxygen species are all around us in the environment as atmospheric ozone (O_3), bleach (HOCl, H_2O_2) and disinfectants (H_2O_2) as well as in some drinking water, tea, coffee and edible oils (H_2O_2) (Aruoma, 1996b). Other reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$), singlet oxygen (O_2) and nitric oxide ($NO^{\cdot}$) are constantly formed in the human body (Aruoma, 1996b). Many of them have useful physiological functions, but they can be toxic when produced in excess (beyond the antioxidant capacity of the body), giving rise to oxidative stress (Aruoma, 1996b). Cellular damage caused by these reactive oxygen species will thus initiate autoxidation in damaged cells (Kitts, 1997).

The relationship between ROS and antioxidants in humans is complex; the protective mechanisms of antioxidants do not act independently but tend to function cooperatively in the form of a cascade (Aruoma, 1996)b. The oxidant-antioxidant balance is an important determinant of immune cell function, not only for maintaining integrity and functionality of membrane lipids, cellular proteins, and nucleic acids, but also for control

of signal transduction and gene expression in immune cells (Meydani *et al.*, 1993). Cells of the immune system and particularly sensitive to changes in oxidant-antioxidant balance because of the higher percentage of polyunsaturated fatty acids in their plasma membranes. Most living organisms possess extremely efficient defense and protective systems in these cells that are essential for defending the organism against oxidative stress induced by ROS (Ramarathnam *et al.*, 1995). However, the capability of such protective systems gradually decreases with age, resulting in disturbances in the normal redox equilibrium that is established in healthy systems (Ramarathnam *et al*., 1995). Such disturbances lead to the malfunctioning of the vital organs, the gradual loss of immunity to disease that tends to be associated with aging, and eventually to death (Ramarathnam *et al.*, 1995). The endogenous antioxidants distributed and around living cells, which regulate the various oxidation-reduction reactions, are therefore seen as a potential class of determinants of longevity (Ramarathnam *et al*., 1995).

A major class of antioxidants responsible for disease prevention is the antioxidant vitamins. The role of the major antioxidant vitamins viz. vitamin E, vitamin C and beta-carotene are reviewed in Chapter 2.6. Photochemical dietary components may also actively contribute to the control of oxidative reactions and provide protection *in vivo* (Furst, 1996). It is therefore conceivable that the mixture of these compounds found in fruits and vegetables can, at the appropriate doses, perform as effective free-radical quenchers, protective antioxidants and/or protectors and regenerators of antioxidants (Furst, 1996). Flavonoids and other phenolic compounds found in plants have received much attention as preventors of human degenerative diseases (Aruoma, 1996a). Studies such as the Zutphen Elderly Study in the Netherlands, in addition, have found beneficial effects of dietary flavonoids from tea and fruit on coronary heart disease (Haumann, 1994). Among other protective activities, phenols scavenge peroxyl radicals, protect against lipid peroxidation and subsequently limit the breakdown of membrane lipids (Aruoma, 1996a). Most importantly, they also control the release of ROS from macrophages and neutrophils by regulating enzymes such as NADPH oxidase (Aruoma, 1996a). Phenolic compounds also exhibit anticarcinogenic activity in numerous animal

models. The anticarcinogenic activity has been correlated with the inhibition of colon, oesophagus, lung, liver, mammary, and skin cancers (Decker, 1995).

In designing antioxidant drugs and/or supplements for therapeutic purposes, it is important to consider the physiological state of the individual consuming the antioxidant. The bioavailability of antioxidants is determined by their pharmacokinetics, which in turn are determined by the degree of absorption, metabolism distribution and subsequent elimination (Aruoma, 1996a). The amount of the antioxidant supplement/drug that becomes available depends on the chemistry of the molecule, the effect of other nutrient components/drugs in the antioxidant cocktail diet, and more importantly, on the physiological state of the consumer (Aruoma, 1996a).

2.7.3 Cosmetic Industry

Skin ageing is a natural multifactorial process (Carletto & Nicolay, 2000). Apart from genetically programmed cell ageing, different external aggressors such as solar radiation, pollution and stress, can promote it (Carletto and Nicolay, 2000). Tissue damage owing to such environmental stimuli results from various oxidative processes, thus this so-called 'oxidative species' involves a large number of chemical reactions and very diverse oxidizing species (Carletto and Nicolay, 2000). These species, which are often oxygen-centered free radicals, are probably mediated via intracellular chromophores or photosensitizers (Jentzsch *et al*., 2000). They have a detrimental effect on all molecular components of the skin cells and the extra cellular matrix. Thus, DNA, proteins and lipids are all affected (Jentzsch *et al*. 2000). Since the main factor of the extrinsic aging is sun exposure, it is often termed photoaging. The clinical changes that are seen in photoaged skin differ from those of normally aged skin in some protected sites. There is increased wrinkling, elastosis, solar comedones, pigmentory changes, and precancerous and cancerous skin lesions (Wester & Maibach, 1997). Within the spectrum of sunlight, UV light is ultimately the major factor leading to photodamaged skin (Jentzsch *et al*., 2000). The uncovered parts of the skin are permanently exposed to UV irradiation-especially in the UVB (280-320nm) range, which leads to the formation of reactive oxygen species

(van Henegouwen *et al*., 1992). Peroxides are implicated in the aging process, and one of their breakdown products, malondialdehyde has been shown to crosslink collagen. Such an effect would explain the decreased elasticity of aged skin (Wester and Maibach, 1997).

Generally, there are two antioxidant defense systems in the skin. The one is a nonenzymic system including alpha-tocopherol and ascorbic acid, and the other is enzymic system, including superoxide dismutase (Hughson, 2002). UVB significantly decreases the enzymic and non-enzymic antioxidants in the skin, thus impairing its ability to protect itself against free radicals generated by exposure to sunlight (CTFA SA Newsletter, 2000). This complex antioxidant defense system has evolved in the skin to protect against ROS; However, UV-generated ROS at least at high levels may substantially compromise the antioxidant defense of the skin, thus tilting the balance towards a pro-oxidant state (Poswig *et al*., 1999). The resulting oxidative stress causes damage to cellular components and changes the pattern of gene expression, finally leading to skin pathologies such as skin cancer, phototoxically, and photoaging (Poswig *et al*., 1999).

The use of antioxidants in cosmetics has a long history. The technology to deliver truly functional antioxidants therefore continues to improve and the ability to neutralize different types of free radicals continues to expand (Madley, 2000). Two different modes of application of antioxidants can be distinguished. Firstly, they are used to stabilize the cosmetic formulation or to protect the skin (Jentzsch *et al*., 2000). As a formulation stabilizer, they serve as a "technical antioxidant". Here they are being used to prevent the oxidative deterioration of labile ingredients such as unsaturated fatty acids or oxidative prone active ingredients (Jentzsch *et al*., 2000). Both synthetic compounds like BHT and natural antioxidants like alpha-tocopherol are frequently used for this purpose (Jentzsch *et al*., 2000). Secondly, as a skin protectant, they serve as a "biological antioxidant". In recent years, this second application mode has become increasingly important. The prevention of oxidative processes within the skin is therefore an additional function of antioxidants in skin care cosmetics (Jentzsch *et al*., 2000).

The basic focus for anti-aging right now is the antioxidant route. Photo protection of the skin can be achieved by the application of tocopherol. This has been shown *in vitro* for the topical application on mouse skin (Jentzsch *et al*., 2000). The combination of tocopherol with ascorbic acid was found to be photo-protective after oral application (Jentzsch *et al.*, 2000). Recent studies have provided strong evidence that certain vitamins, such as vitamins E and C which are also antioxidants can augment the performance of UV filters and thus provide the optimum broad spectrum protection against environmental stress and premature skin aging (CTFA SA Newsletter, 1999). Topical vitamin E treatment significantly reduced the formation of lipid hydroperoxides in the skin after UV irradiation (Lopez-Torres *et al*., 1998). Tocopherol acetate, commonly known as vitamin E acetate, the more stable form of vitamin E, prevents skin damage by free radicals and protects the skin against UV damage (Hughson, 2002). After topical application during animal studies, vitamin E has been reported to be useful in reducing cell membrane phospholipid peroxidation (Wester and Maibach, 1997). In animal studies, vitamin E has been reported to be useful in reducing UV light damage as determined by the decrease of ornithine decarboxylase in the skin (Wester and Maibach, 1997). These results suggested that the topical administration of vitamin E might protect the skin through a mechanism that involves the up-regulation of a network of enzymatic and non-enzymatic reactions.

Highly reactive antioxidants such as vitamin C and its derivatives are actives that play an important role in the skin-lightening process by competing with melanin precursors for the oxidative stress available to the system (Hughson, 2002). The antioxidant effect of ascorbyl phosphates has the additional advantage of protecting the skin against UV damage (Hughson, 2002). Vitamin C, a powerful antioxidant and potential wrinkle reducing ingredient, when topically applied, functions as a biological cofactor and free radical scavenger, which inhibits the onset of UV-induced erythema and skin damage, alleviating the effects of natural vitamins loss from the body to a large degree (Knowlton, 2001). Co-enzyme Q10 is a very powerful antioxidant naturally present in the intracellular region of the dermal and epidermal layers and is thought to function by

reducing the formation of intracellular peroxides that are induced by oxidative stress in skin cells (Knowlton, 2001).

Natural antioxidant extracts have also been used successfully in the cosmetic industry. Some scientific discoveries that help to slow down the formation of fine lines and wrinkles include the identification of certain fruit acids from plant extracts, the antioxidant properties of common herbs such as sage, parsley and thyme, and the ability of some essential oils to repair damaged skin (Earle, 1993). One of the latest natural extracts to be researched is grapeseed extract, whose relatively high polyphenol content is said to exhibit excellent antioxidant properties, protecting the skin from the devastation of premature aging (Knowlton, 2001). Green tea provides antioxidant benefits and stimulated the formation of collagen and elastin (Tew, 2002). Exotic plants like suma, ginkgo biloba, golden seal, dong quai, gotu kola and angelica may exhibit antioxidant properties in cosmetic formulations (Daswani, 1996). These plants contain vitamins, minerals, and other substances known to neutralize free radicals. Even the common marigold flower (also known as calendula) contains to several natural chemicals capable of slowing the body's aging processes (Daswani, 1996). Other possible sources of antioxidants include ivy, rosemary, chamomile, papaya, goldenseal, ginseng, watercress, and comfrey (Daswani, 1996).

2.8 PLANT MATERIAL FOR STUDY

2.8.1 Sage *(Salvia officinalis)*

Family: Labiatae

Figure 2.16: Sage (*Salvia officinalis*)

2.8.1.1 Characteristics

Sage, a universally used flavouring herb, originates from the North Mediterranean coast. A hardy perennial shrub, sage can grow to a height of 1 m/40 inches tall with a 30 cm/12-inch spread (Craig and Harris, 1998). The somewhat woody stems branch into long oval grayish leaves (Figure 2.16). Flowers appear around midsummer. These range from white to blue and pink, and grow in vertical clusters of small, tubular blooms (Garland, 1995; Craig and Harris, 1998). The flowers are generally deep throated and attractive to bees (Garland, 1995). Sage has a particularly appealing scent and a slightly warm flavour with a hint of camphor (Craig and Harris, 1998). It is one of the few herbs whose flavour strengthens when it is dried, and since it has a powerful flavour, the dried and ground versions should be added discretely (~~Ortiz 1992~~Garland, 1995).

2.8.1.2 Uses

Sage is a traditional ingredient of stuffing, and a delicious accompaniment to poultry dishes. It is also a classic *bouquet garni* mainstay (Craig and Harris, 1998). Leaves can also be brewed into a relaxing tea. They have a strong, dry, pungent flavour with a bitter tang that is particularly welcome with fatty foods. Sage leaves are used in sausages and in stuffings for goose and pork and as flavouring in cheeses, such as Sage Derby and Vermont Sage cheeses, and in rich eel dishes (Garland, 1995). Although uncommon in French cookery, sage is often used in Italy with veal and game and in the Middle East it is threaded with lamb and onions in kebabs (Garland, 1995).

A sage tea can ease sore throats and gums, either drunk or as a mouthwash. It can also relieve indigestion and minor upset stomachs (Garland, 1995; Craig and Harris, 1998). Sage should not be taken to excess, as it can have harmful effects, and should not be taken as a medicine by pregnant women (Craig and Harris, 1998). It has an astringent and tonic action, stimulating digestion, cooling fevers and cleansing the blood (Garland, 1995). The disinfectant action has led to its use as a fumigant. An infusion of the leaves is

very pleasant and relaxing (Garland, 1995). It helps to ease headaches, nervous tension and to promote menstruation (Garland, 1995). To revitalize and darken thin or graying hair, a strong infusion of sage can be used as a regular rinse (Garland, 1995).

Sage is reported to one of the most potent spices as natural antioxidants, among spices prompting a great deal of experimental work aimed at isolating and identifying the compounds responsible for the antioxidant activity (Rajalakshmi and Narasimhan, 1996). High content of phenolic compounds was established in various *Salvia* species (Millauskas *et. al.*, 2004). Carnosol (an odourless and tasteless phenolic diterpenic lactone) and carnosic acid have been found in sage (Madsen and Bertelson, 1995; Pokorny, 1991).

2.8.2 Oregano *(Origanum vulgare)*

Family: Labiatae

Figure 2.17: Oregano *(Origanum vulgare)*

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2.8.2.1 Characteristics

Oregano is a wild variety of marjoram (Figure 2.17). A half-hardy perennial, oregano can grow to around 45 cm/18 inches tall, and spread to about half this (Craig and Harris, 1998). Oregano has a long, green, somewhat woody stalk, which branch into delicate, oval leaves (Craig and Harris, 1998). Flowers bloom around the end of summer, and are clusters of tiny white or pink species. The flowers are very attractive to bees and butterflies (Garland, 1993). Oregano has a delicate scent, and a sweetly spicy flavour (Craig and Harris, 1998). Oregano is tall, vigorous and leggy, spreading by rhizomes among grasses on dry banks and stony hillsides (Garland 1995). When grown in hot, dry conditions the flavour is far stronger, more peppery and has a bitterer tang (Garland, 1995).

2.8.2.2 Uses

Oregano plants should be cut as soon as flowering begins (Craig and Harris, 1998). Leaves can be snipped individually for use fresh, while whole stems should be gathered for drying (Craig and Harris, 1998). Oregano should, however, be used with discretion in the kitchen. Fresh leaves should be added shortly before the end of cooking for the best flavour (Craig and Harris, 1998). They are delicious added to soups and pasta dishes or as

a seasoning to most meat recipes (Craig and Harris, 1998). These herbs are native to the Mediterranean region, appearing in many French and Italian dishes especially tomato-based sauces (Ortiz, 1992). In Italy, oregano is one of the most popular flavouring herbs for pizzas, pasta and all tomato and cheese dishes (Garland, 1995). In Mexico it flavours chillies, spicy soups and bean dishes (Garland, 1995).

It is the wild species of oregano that is chiefly used as a medicine and has had a reputation since classical times as an antidote (Garland, 1995). Oregano can be used as a stimulant, carminative, stomachis, diuretic, and adiaphoretic. An infusion can relieve bronchial congestion, menstrual cramps or indigestion, and can be applied externally to reduce the pain of sprains or stiff joints (Craig and Harris, 1998). Its aromatic oil has an antiseptic effect, is mildly tonic and digestive, will provoke menstruation and soothe and calm the nerves (Garland, 1995). An infusion will settle the stomach and is helpful in cases of morning sickness (Garland, 1995). The essential oil of oregano has antibacterial activity against *Listeria* and *Staphylococcus*.

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Oregano has reported as being a effective antioxidant. Five different phenolic compounds have been isolated from the methanol extract of oregano (*Origanum vulgare* L.); all five showed antioxidant activity (Madsen and Bertelsen, 1995). The five antioxidant phenolic acids are: phenyl glucoside, protacechuic acid, caffeic acid, rosmarinic acid and a new compound established as 2-caffeoyloxy-3-{2-(4-hydroxybenzyl)-4,5-dihydroxy} phenylpropionic acid (Six, 1994). One of the compounds was identified as rosmarinic acid (Madsen and Bertelsen, 1995). In addition, carvacrol and thymol have been isolated from the essential oil of oregano (Madsen and Bertelsen, 1995). Oregano showed antioxidant activity in stabilizing lard against oxidation (Vekiari *et al.*, 1993).

2.8.3 Coriander *(Coriandrum sativum)*

Family Umbeliferae

Figure 2.18: Coriander (*Coriandrum sativum*)

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2.8.3.1 Characteristics

A hardy annual, growing to 60 cm/24 inches and with a spread of 22 cm/9 inches, coriander leaves are flat and fern-like (Figure 2.18), similar in appearance to some types of parsley (Craig and Harris, 1998). The flowers bloom in midsummer in small, pink-tinged umbels and are followed by large round seeds (Garland, 1995). An aromatic plant, the leaves have a sharp taste, while the seeds are sweeter, and spicier (Craig and Harris, 1998).

2.8.3.2 Uses

Coriander leaves should be gathered when young-usually when the plant is around 15 cm/6 inches tall. They are best stored frozen rather than dried (Craig and Harris, 1998). Seeds should be fully ripe before harvesting. Native to Southern Europe as well as the Middle Far East, this ancient annual herb, a member of the carrot family, is one of the most popular herbs in cuisines around the world (Garland, 1995). Leaves are commonly used in curries, mexican dishes such as salsas, chutneys, or fresh in salads (Craig and Harris, 1998). Seeds also feature in curries and casseroles, and have a particularly piquant flavour when roasted (Craig and Harris, 1998). The root can be cooked as a vegetable, although the flavour may not be everyone's taste (Craig and Harris, 1998). As the seeds

ripen they become strongly aromatic, sweet and pleasant, with a slightly bitter edge like zest of orange (Garland, 1995). They can be used to flavour both sweet and savoury food, liqueurs and sweetmeats, stews, pickles and marinades and especially curries. Eaten on their own, they do not suit some palates, having a strong, almost bitter taste, but when combined with chillies and other hot foods, they provide a cool, pungent and unique flavour to the hot spices (Garland, 1995).

~~Although not used so much in herbal medicine, coriander~~Coriander functions medically as a carminative, diuretic, tonic, stimulant, stomachic, aphrodisiac, analgesic and anti-inflammatory agent. Coriander seeds can be chewed to aid digestive complaints, and a poultice of bruised seeds can relieve joint pain and also haemorrhoids (Craig and Harris, 1998). The first monks included coriander among their medicinal herbs. Today coriander seeds are occasionally used to disguise the taste of other unpleasant medicines and the aromatic oil in the seed acts as a stimulant and digestive (Garland, 1995).The essential oil of the seed saponins of coriander has antifungal activity against *Ascophaera~~s~~ apis* and *Fusa~~e~~rium oxysporu*-s.

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2.8.4 Fenugreek *(Trigonella foenum-graecum)*

Family Leguminosae

Figure 2.19: Fenugreek (*Trigonella foenum-grecum*)

2.8.4.1 Characteristics

Fenugreek is a handsome annual that grows about 60 cm/2ft bearing yellowish pea flowers in midsummer. It has trifoliate leaves (Figure 2.19) and dramatically long narrow pods containing at least 10 square seeds, reaching maturity in a few months in warm climates (Garland, 1995).

2.8.4.2 Uses

In cooking, fenugreek seeds must be dry-roasted before use to remove the bitter flavour, although over roasting will also result in an unpleasant taste (Garland, 1995). Ground fenugreek is an essential ingredient in curry powders and is also used for pickling. In parts of Africa, the seeds are soaked and prepared like legumes (Garland, 1995). The seeds can also be sprouted and added to green salads, where they add a crunchy texture and a slightly bitter flavour (Ortiz, 1992). Fenugreek is best known as a powdered ingredient, but is also used for confectionery and as a pickle flavouring (Garland, 1995).

Fenugreek seeds are rich in vitamins, nitrates and calcium, have a softening, soothing action and are said to encourage lactation (Garland, 1995). The seeds can be infused in water for a tonic tea, or apply soaked seeds can be applied as a poultice for inflammations of the skin (Garland, 1995). Fenugreek has carminative, tonic and aphrodisiac properties. The seed can also be used as an insect repellant.

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CHAPTER 3
MATERIALS & METHODS

3.1 MATERIALS

3.1.1 Sample Preparation

The sample materials used were four common culinary/spice herbs: sage, oregano, coriander and fenugreek. Sage and oregano leaves were obtained commercially in a dried, ground form from Spice Emporium, Durban. Coriander and fenugreek were obtained fresh from the Durban local fresh produce market. The leaves of the coriander plant and the leaves and stems of the fenugreek plant were dried in a vacuum oven at 50 ^{o}C. This process of slow drying prevented the degradation of the nutritive components of the herbs. The drying times of the herbs varied according to the texture of the herb as well as the amount of moisture, which was assessed visually. Once dried, the dried herbs were ~~accumulated and thereafter~~ ground in a food processor. The ground herbs were stored in airtight containers until further use.

3.2 METHODS

3.2.1 Determination of Concentration of Vitamins in Herbs

The concentrations of Vitamin C, Vitamin E and Beta carotene were determined for each of the herbs by specific methods.

3.2.1.1 Determination of Vitamin C

The concentration of vitamin C in the herbs (sage, oregano, coriander and fenugreek) was determined by a titrimetric method (Association of Vitamin Chemists, 1947). This

procedure was based on the oxidation reaction of ascorbic acid to dehydroascorbic acid. Reagents: meta-phosphoric acid, phenol-indo-2:6 dichlorophenol indicator, vitamin C standard were prepared as per Appendix C.

a. Standardization of Vitamin C solution

Two millilietres of the standard vitamin C (ascorbic acid) solution and 5 ml meta-phosphoric acid (M.P.A) solution were dispensed into a 250-ml volumetric flask. The mixture was titrated with the indicator solution until a pink colour persisted for 15 seconds. The titrations were carried out in triplicate and the average of the three determinations was calculated. A blank titration was also carried out in which 7 ml M.P.A solution and 3 ml water were titrated with the indicator solution as in the standardization procedure.

b.Vitamin C Content of Herb

Ten grams of each of the dried herbs were weighed accurately into a 100-ml volumetric flask and made up to mark with M.P.A solution. This was then well shaken for at least 2-3 minutes and thereafter filtered through a Whatman No. 4 filter paper. 10 ml of the filtrate was then titrated with the indicator solution

~~as in the above discussed procedures.~~ The titre volumes of the samples and standard vitamin C were then used to calculate the vitamin C concentrations of the herbs.

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c. Calculation:

$$\text{Vitamin C (mg/100g)} = \frac{\text{titre of sample} \times 2 \times 100 \times 0.1 \times 100 \times 1000 \text{ mg}}{100 \times 10 \text{ ml} \times (\text{A-B}) \times \text{sample weight (g)}}$$

Where A=Average titre of standard

B=Average titre of blank

3.2.1.2 Determination of Vitamin E

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Vitamin E content of the herbs was determined using the method outlined by Roche (1989). This method involves the saponification of the herbs, followed by extraction of the unsaponifiable material and then determining the Vitamin E concentration by High Performance Liquid Chromatography (HPLC) using a reverse phase C18 column.

a. Alkaline Saponification of Herbs

Five grams of each sample material (sage, oregano, coriander and fenugreek) was measured accurately and transferred to 250-ml round-bottom flask fitted with a ground stopper. The sample was mixed with 50 ml of methanol-ascorbic solution (0.5 g pure, crystallised ascorbic acid in 4 ml warm, distilled water, mixed with 20 ml ethanol and diluted with methanol to 100 ml). This solution was mixed until the sample material was moistened, following which it was brought to boiling point on a water bath, while purging with nitrogen gas. 5 ml of a 50% (w/v) potassium hydroxide solution was added, and the mixture thoroughly mixed. The mixture was then saponified at boiling point for 20 minutes under nitrogen reflux. The mixture had to be shaken from time to time to prevent any material from adhering to the sides of the flask.

~~d.~~ b. Extraction of Unsaponifiable Material

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On completion of the saponification, the contents of the flask were rinsed with a minimum amount of water (no more than 30-50 ml in two or three portions) into a 500-ml separating funnel, and the saponification flask was subsequently washed with 80 ml of diethyl ether. The warm mixture was allowed to expand (in such conditions emulsions can be largely avoided) several times before the actual ether extraction. The extraction was repeated two more times with 80 ml diethyl ether. The combined ether phases were washed with distilled water until neutral pH, and then transferred to a 250-ml volumetric

flask. The separating funnel was rinsed with approximately 20 ml diethyl ether. The volume was made up to 250 ml with diethyl ether.

c. High Pressure Liquid Chromatography

An aliquot had to be taken containing approximately 0.1 mg alpha-tocopherol (calculated on the basis of the declared content of the sample and the weighed sample) from the ether extract and evaporated to dryness. For the first 4 samples, a 800 µl ether aliquot was evaporated down with nitrogen, then reconstituted with 10 µl Vitamin A internal standard in ethanol (Vitamin A concentration: 400 µg/ml), 40 µl methanol and 50 µl ether. 25 µl of this solution was injected into the columns. For the last two samples, a 2000 µl ether aliquot was evaporated down with nitrogen.

The HPLC that was used was a Hewitt Packard 1090. The conditions for HPLC were as follows:

- Column: Waters Spherisarb SS ODS2
- Stationary phase: C18
- Mobile phase: Methanol: 98%, H_2O: 2%
- Flow rate: 1ml/min.
- Pressure: ±100 bar
- Temperature: 40^oC
- Injection volume: 25µl
- Wavelength: 292 nm
- Detection: UV
- Run time: 13 min.
- Standard solution for external calibration: 95 µg/ml Vitamin E

The parameters measured were used to calculate the concentration of vitamin E in the herbs.

Calculation:

$$\frac{\text{Test (area counts)}}{\text{Internal std (area counts)}} \times \frac{\text{concentration of Internal std. (µg)}}{\text{rf value (Vit E std/Vit A std)}} \times \frac{1}{0.59} \times \frac{1\,000}{200}$$

.

= µg/ml x 100= µg/dl x 10/430/7 = mg/kg vitamin E

3.2.1.3 Determination of Beta-Carotene

The procedure (Indian Journal of Nutrition and Dietetics, 1967) to determine the concentration of beta-carotene involved the alkaline saponification of the sample, extraction of the carotene with diethyl ether, open-column chromatography on aluminium oxide and spectrophotometric assay of the carotene fraction; calculated as the β-carotene equivalent. The materials to be analyzed were the dried herbs (sage, oregano, coriander and fenugreek) ground to a fine powder.

a. Saponification of Sample Material

Five grams of each of the finely ground and mixed sample was weighed into a 250-ml round-bottom flask. Sixty millilitres of ethanol, 10 ml of a 50% (w/v), aqueous potassium hydroxide solution and 10 ml of petroleum benzene was added to the flask containing the sample material. A nitrogen atmosphere was established and the sample was saponified by heating it in a water-bath for 30 minutes under reflux conditions (water-bath temperature about 50^0C). Thereafter the reaction mixture was cooled to room temperature, following which the reaction mixture was transferred quantitatively into a 500-ml separating funnel. The empty flask was flushed with 10 to 20 ml distilled water followed by 100 ml diethyl ether, poured into the same separating funnel. The mixture was shaken vigorously to extract the carotene, and the mixture was thereafter allowed to stand to separate the phases. The aqueous phase was then transferred into a second separating funnel and extracted out again with 100 ml of diethyl ether. The ether phases

were pooled, washed with distilled water to neutral pH and evaporated to dryness in a rotary evaporator at 50 ^{o}C. Addition and evaporation of absolute ethanol removed the last traces of water.

b. Preparation of Aluminium Oxide Column

Hundred grams of aluminium oxide (neutral grade, activity level 1) was weighed into an Erlenmeyer flask with a ground stopper. 12 ml of distilled water was added to this and the flask was immediately closed with the stopper. The solution was shaken until all lumps were broken and this mixture was allowed to stand at room temperature for 1 hour before use. This preparation could be stored for a maximum of 24 hours. The 'ready-for-use' deactivated aluminium oxide was suspended in n-hexane and this suspension was slowly poured into a chromatography tube until the column of aluminium oxide sediment was 5-7 cm high. The chromatography tube that was used had an inside diameter of 6-20 mm, and was fitted with a stopcock and glass filter. The column was kept covered with *n*-hexane at all times.

~~c.~~ c. Open-Column Chromatography

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The evaporation residue (from the saponification step) was taken up in about 1- 2 ml *n*-hexane and pipetted quantitatively on to the prepared aluminium oxide column. The column was washed with n-hexane until the carotene zone could be seen at the bottom of the column. The *n*-hexane eluate was collected in a 50-ml round bottomed flask and the eluting was continued until the eluate became colourless. The eluate was evaporated to dryness in a rotary evaporator (at 50^{o}C), and the residue was redissolved in 0.2 ml chloroform and diluted with an appropriate volume of cyclohexane (depending on the expected amount of carotene). The absorbency of the solution was then measured in a 1-cm cuvette against that of a blank cyclohexane at about 456 nm (at the absorbance maximum) in a spectrophotometer. The absorbance ~~cies obtained were then~~was used to calculate the beta-carotene concentrations of the herbs.

d. Calculation:

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$$\text{mg carotene/ kg sample} = \frac{\text{Abs.} \times 1000 \times \text{Vol.} \times 1000}{2500 \times 100 \times \text{sample weight (g)}}$$

Abs. = Measured absorbance at ca. 456 nm (at the maximum)

Vol. = Volume of the assay solution

2500 = E (1 %/ 1 cm)

Theoretical absorbance of a 1% solution (w/v) of β-carotene measured in cyclohexane at about 456 nm in a 1-cm cuvette.

These results were recorded in mg carotene as the β-carotene equivalent (sum of α-, β-, and γ-carotene).

3.2.2 Analysis of antioxidant activity of herbs

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Two different methods were used to analyze the antioxidant effect of the herbs. The first method was the beta-carotene bleaching method which determines the antioxidant response time. The second method was by the utilization of the Rancimat apparatus which measures induction periods. Induction periods were then used to calculate the antioxidant index which was used as a measure of antioxidant activity.

3.2.2.1 Measurement of Antioxidant Response via Beta-carotene Bleaching Method

a. Preparation of Beta-carotene Emulsion

Sixty milligrams of crystalline beta-carotene (1%), 1.0g linoleic acid, and 2.0 ml Tween 60 were dissolved in 20 ml chloroform. Due to its viscosity, the Tween 60 was warmed in a water bath at 50°C before pipetting. The chloroform was removed at 40°C under vacuum using a rotary evaporator. The resulting viscous red oil was immediately diluted with triple distilled water (single distilled water passed through a mixed-bed ion

exchange resin column can also be used) to 25 ml in a volumetric flask and was thoroughly mixed. The emulsifiable concentrate could be held in the dark at room temperature as long as 1.5 hours. Pure oxygen was bubbled through triple-distilled water for 0.5 hours. The 25 ml emulsifiable concentrate was poured into 500 ml of the oxygenated water using vigorous magnetic stirring. This diluted emulsion had to be used immediately.

b. Measurement of Antioxidant Response

Antioxidant response is the induction time in the progress of carotene destruction.

i. Antioxidant response of herbs

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The solutions of the herbs (sage, oregano, coriander and fenugreek) were prepared with ethanol at a 7.5 µg/ml concentration. A 0.1 dilution was made immediately using absolute ethanol. A 2.0 ml aliquot of antioxidant solution was placed in the large test tubes to provide 15 µg of antioxidant per tube. The control tube contained 2.0 ml absolute ethanol, 50 ml of the diluted, oxygenated emulsion was added to each tube, glass stoppers were attached, and the tubes were inverted several times for thorough mixing. The stoppers were removed and the tubes immediately placed in racks in a 50^0C water bath. At 15-minute intervals, 0.5 ml aliquots from each tube were pipetted directly into the spectrophotometric tubes containing 2.5 ml 95% ethanol. Readings were made against a 95% ethanol blank with the wavelength set at 470 nm. A zero time reading was taken when the diluted emulsion was pipetted into the large test tubes. Absorbence values that were obtained of the solutions were used as a means of analysis when plotted on a graph with the time interval used as the independent variable. The Antioxidant Response was calculated using the following equation:

Antioxidant Response (%) = $(\text{Absorbence}_a - \text{Absorbence}_c) \times 100$

a= antioxidant (herbs), c= control

ii. Antioxidant response of combinations of herbs

The same measurement procedure was used as for the individual herbs as discussed above, but the sample solutions were prepared with a 7.5 µg/ml concentration of 1:1 ratios of the following combinations of herbs: sage + oregano, sage + coriander, sage + fenugreek, oregano + coriander, and coriander + fenugreek. The absorbance values measured were used to determine antioxidant response.

3.2.2.2 Measurement of Antioxidant Index via Rancimat Induction Period Method

The Rancimat apparatus was used to measure the induction periods of the herbs in the substrate oil. The induction periods was used to calculate the antioxidant response.

a. Optimization of Method

Apparatus: Metrohm Rancimat Model 679 with cleaned glassware

Instrument temperature: 120^0C

Chart speed: 1 cm/hour

Airflow rate: 20l/hour

Figure 3.1: Rancimat 679

Prior to the use of the Rancimat, it was imperative that all glassware was thoroughly cleaned as per the operating instructions in the 679 Rancimat (Figure 3.1) operating manual. A 2.5 g sample of substrate oil (Appendix D) was weighed accurately into each

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of the reaction vessels. The vessels were then placed in the heating block of the wet section. The reaction vessels were then connected to the measuring vessels in the wet section. 60 ml of deionised water was measured into each of the measuring vessels, containing the electrodes. The measuring vessels were also placed in the wet section. All parts were connected to the apparatus as per the operating instructions, and the test was carried out until the endpoints of all the samples were reached, with a maximum limit of 48 hours allowed.

b. Measurement of Induction Periods

i. ~~Herbs~~Induction periods of herbs

For the evaluation of the effectiveness of the antioxidants in the herbs in stabilizing the substrate oil, various concentrations of the herbs and combinations thereof were dispersed in 2.5 g of the substrate oil and placed in the reaction vessels. The measurement procedure was the same as in Section 3.2.2.2 a. The herbs that were used as sample material had to be finely ground for analysis. Individually, the herbs were used in 20%, 40%, 60% and 80% (w/w) concentrations. ~~In combinations, the herbs were used in a 1:1 ratio at 40% and 80% (w/w).~~ The graphs automatically printed from the Rancimat extrapolate the induction periods which was used to calculate the antioxidant index.

ii. ~~Antioxidant Vitamins~~

~~A control study was conducted to assess the antioxidant activity~~**Induction periods of** ~~standard antioxidant vitamins. For the assessment~~**combination of** ~~the potency of the vitamins on the stabilization of the stripped sunflower oil, the vitamins were added at various concentrations to 2.5 g substrate oil. The vitamins that were analyzed included the antioxidant vitamins: vitamin E (alpha-tocopherol), vitamin C and beta-carotene. The concentrations of the vitamins were 250 ppm, 500 ppm, 750 ppm, 1000 ppm and 1250 ppm. In studying the cumulative effect of the antioxidant vitamins, alpha-tocopherol (250~~

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~~ppm) was combined with vitamin C and beta-carotene (25 mg and 50 mg concentrations). Measurement procedures were same as in Section 3.2.2.2 a.~~**herbs**

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The same measurement procedure discussed above for individual herbs was used. In combinations, the herbs were used in a 1:1 ratio at 40% and 80% (w/w). The graphs automatically printed from the Rancimat extrapolate the induction periods which was used to calculate the cumulative antioxidant index.

~~c.~~*d. Determination of Antioxidant Index*

Processing and the evaluation of the recorded induction periods are performed in the 679 Rancimat automatically. The original experimental values used for plotting the curves are subjected to a smoothing procedure for the evaluation.

The increase or decrease in antioxidant index value measured the optimum level of each herb and their cumulative antioxidant activity as in equations (a) and (b):

a. Antioxidant index value = $\frac{\text{Induction period of oil substrate with antioxidants}}{\text{Induction period of oil substrate}}$

b. Cumulative antioxidant index value =

$$\frac{\text{Induction period of oil substrate with mixed antioxidants}}{\text{Induction period of oil substrate}}$$

3.2.2.3 Control Experiment: Antioxidant Index of Antioxidant Vitamins

A control experiment was conducted whereby antioxidant vitamins were assessed for their antioxidant effects, through measurement of antioxidant index. In order to determine ~~the potency of the vitamins on the stabilization of the stripped sunflower oil, the vitamins were added at various concentrations to 2.5 g substrate oi~~~~the potency of the vitamins on the stabilization of the stripped sunflower oil, the vitamins were added at various concentrations to 2.5 g substrate oil~~the antioxidant potency of the vitamins on the stabilization of the stripped sunflower oil, the vitamins were added at various concentrations to 2.5 g substrate oil. Vitamin E (alpha-tocopherol), vitamin C and beta-carotene were tested at concentrations of 250 ppm, 500 ppm, 750 ppm, 1000 ppm and 1250 ppm. The cumulative effect of the antioxidant vitamins was analyzed for combinations of alpha-tocopherol (250 ppm) with vitamin C and beta-carotene (25 mg and 50 mg concentrations). The procedures outlined in Section 3.2.2.2 a. were followed.

3.2.2.4 Determination of the cumulative antioxidant activity of the herbs in a cosmetic preparation

~~The base cosmetic preparation of choice~~The antioxidant effect of the herbs in a cosmetic preparation was analyzed using the Rancimat to determine the antioxidant and the cumulative antioxidant indexes. The base cosmetic preparation used was aqueous cream which was prepared, according to an in-house laboratory standard formulation (Appendix E).

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a. Measurement of Antioxidant Index

Two and a half grams substrate oil (Appendix D) and 2.5 g aqueous cream (Appendix E) ~~were~~ was measured accurately into the reaction vessel. This mixture was mixed before the herbs were added. The herbs were added individually in concentrations of 20%, 40%,

60% and 80% (w/w~~); and for cumulative effect the herbs were combined (1:1 ratio) at 40% and 60% (w/w) concentrations.~~). The measurement procedure was as per Section 3.2.2.2 a.

b.

Measurement of Cumulative Antioxidant Index

To determine the cumulative effect, the herbs were combined (1:1 ratio) at 40% and 60% (w/w) concentrations. The measurement procedure was in Section 3.2.2.2 a.

CHAPTER 4

RESULTS

4.1 CONCENTRATION OF ANTIOXIDANT VITAMINS IN HERBS

Table 4.1 lists the concentrations of the antioxidant vitamins: vitamin C, vitamin E and beta-carotene, in the herbs: sage, oregano, coriander and fenugreek. The concentration of the antioxidant vitamins in the fenugreek was determined in both the stem and leaves, in order to investigate the source of possible nutritional components. Coriander was found to have the highest concentration of ~~aantixidant~~all the vitamins. The vitamin C concentrations (mg/kg) was in the order of coriander > fenugreek (l) > fenugreek (s) > oregano > sage. The Vitamin E concentration were in the order of coriander > sage > oregano >fenugreek (l) > fenugreek (s). The Beta-Carotene ranged from coriander > fenugreek (l) > oregano > sage > fenugreek (s) (Table 4.1). The vitamin concentration of sage and oregano were similar, an indication of the close botanical relationship between these herbs. The leaves of the fenugreek plant had higher concentrations of the vitamins than the stems. This indicates that the leaves of the fenugreek plant are more nutritious than the stems, prompting the use of fenugreek leaves in future investigations as was the case with the other herbs under investigation.

Table 4.1: Concentration of antioxidant vitamins in herbs

Herb	Vitamin C (mg/kg sample)	Vitamin E (mg/kg sample)	Beta-carotene (mg/kg sample)
Sage	279.12	229.24	103.79
Oregano	359.74	227.80	131.75
Coriander	822.25	644.40	577.36
Fenugreek (l)	650.01	142.90	170.21
Fenugreek (s)	431.01	147.00	27.54
Fenugreek (w)	582.11	205.00	116.48

(l) – leaves, (s) – stems, (w) - leaves + stems

4.2 ANALYSIS OF ANTIOXIDANT RESPONSE OF HERBS WITH THE BETA-CAROTENE BLEACHNIG METHOD

Figures 4.1 show the difference in ~~absorbences~~absorbencies between the herbs and control solution, at time intervals of 30, 60 and 90 minutes. The rate of beta-carotene decolourization during 15-minute intervals at 470 nm indicated that the herbs: coriander; sage; oregano and fenugreek showed antioxidant function in decreasing the rate of carotene decolourization (oxidation), by preventing the destruction of carotene by oxidation as compared to the control. All herbs independently had considerable antioxidant functions, in decreasing carotene oxidation (Figure 4.1a). Coriander and fenugreek performed better than sage and oregano (Figure 4.1a). Furthermore, the antioxidant function was higher in the fenugreek leaves, as compared to the stems (Figure 4.1b).

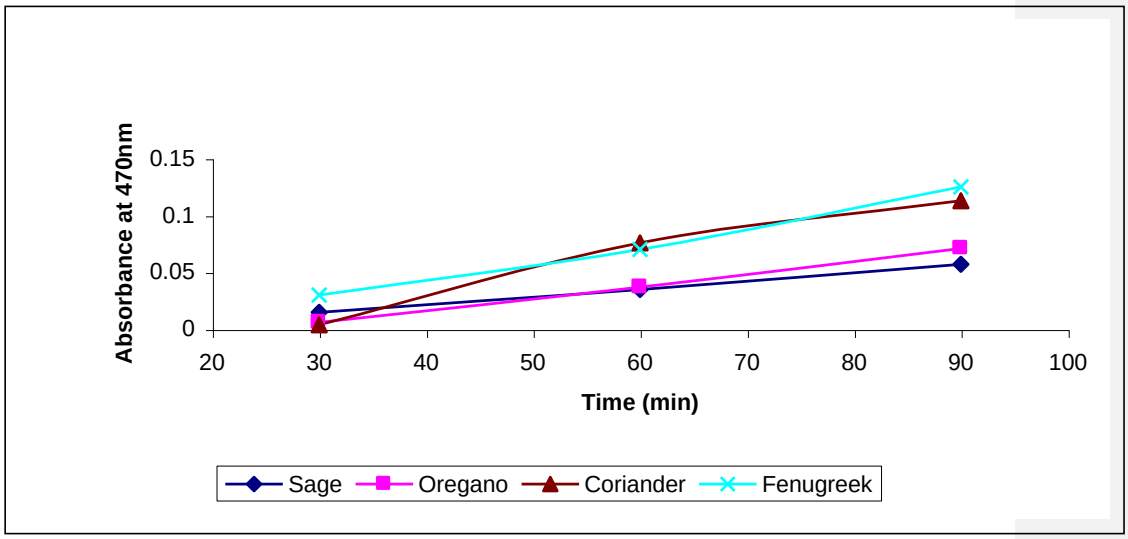


Figure 4.1 (a): The antioxidant effect of ~~selected herbs/botanicals~~ sage, oregano, coriander and fenugreek on the rate of carotene oxidation (n=3)

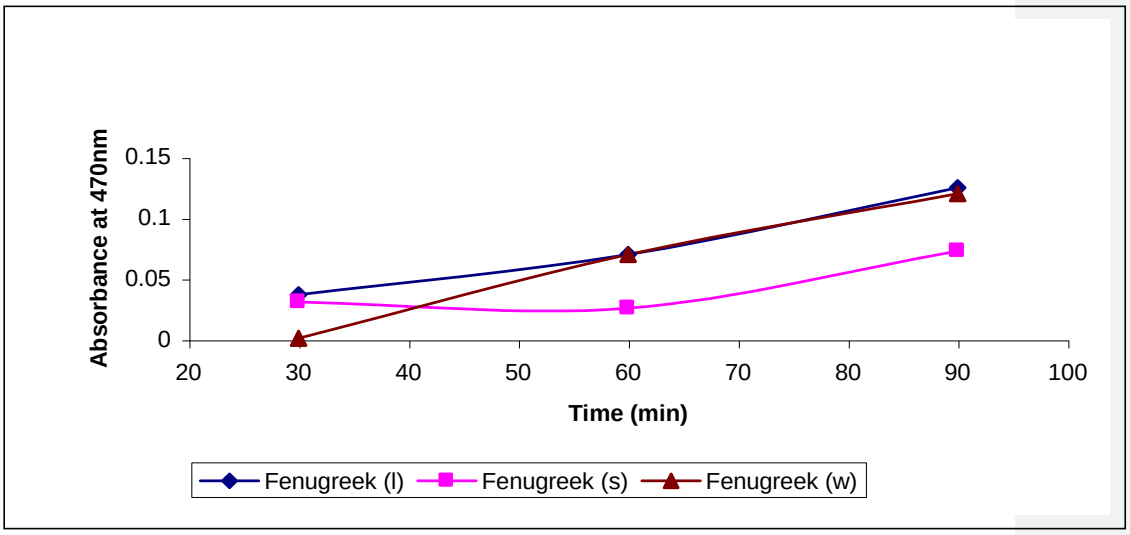
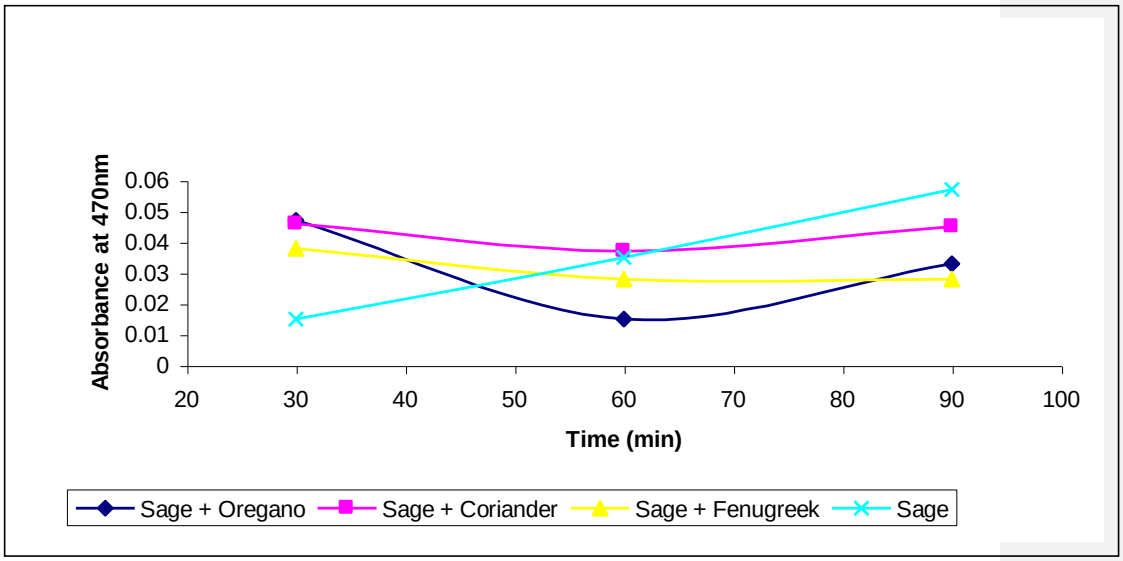
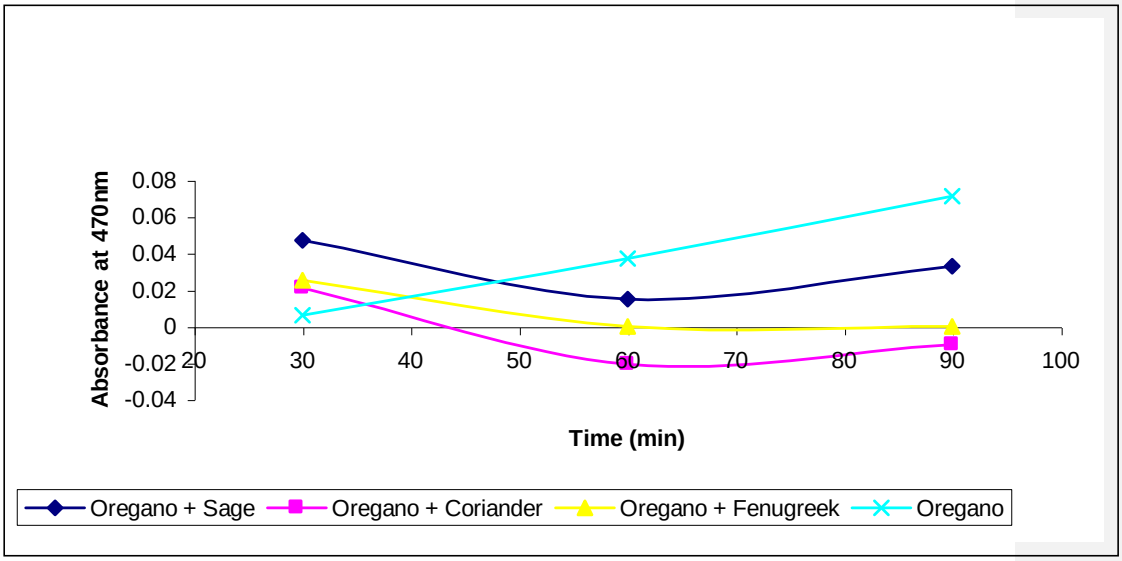


Figure 4.1 (b): The antioxidant effect of ~~selected herbs/botanicals~~ fenugreek on the rate of carotene oxidation (l)-leaves, (s)-stems, (w)-leaves + stems (n=3)

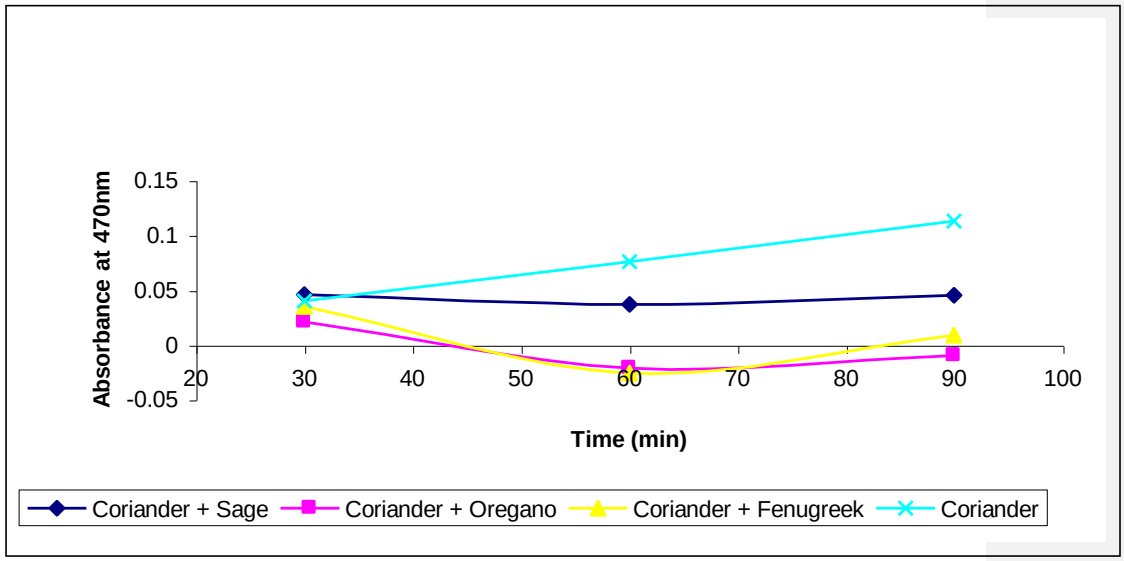
The effect of the combination of the herbs was also evaluated for possible synergistic and complementary interactions (Figure 4.2). As illustrated in Figure 4.2a. coriander was a synergist in enhancing the antioxidant effect of sage, by increasing the antioxidant effect. The other 2 herbs: oregano and fenugreek decreased the antioxidant effect when combined with sage, as compared with sage alone. This was thus indicative of prooxidant effects. Pro-oxidants are compounds that induce oxidative stress and the fact that oregano and fenugreek decreased the total antioxidant effect, they could be functioning as pro-oxidants. Oregano, fenugreek and coriander were more effective as antioxidants alone (Figure 4.2 b-d) than in combinations. No further observations could be made in determining any further possible synergistic and complementary relationships.


Absorbance at 470nm
0.06
0.05
0.04
0.03
0.02
0.01
0
20
30
40
50
60
70
80
90
100
Time (min)
Sage + Oregano
Sage + Coriander
Sage + Fenugreek
Sage

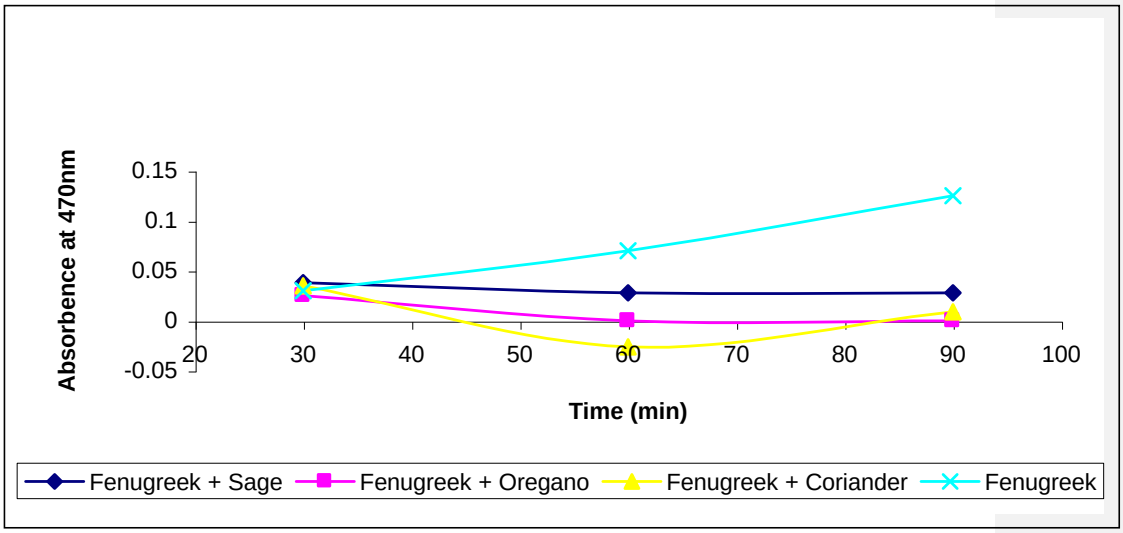
(a)


Absorbance at 470nm
0.08
0.06
0.04
0.02
0
-0.02
-0.04
20
30
40
50
60
70
80
90
100
Time (min)
Oregano + Sage
Oregano + Coriander
Oregano + Fenugreek
Oregano

(b)



(c)



(d)

F

igure 4.2: The~~effect~~ antioxidant effects of the combination of sage (a), oregano (b), coriander (c) and fenugreek (d) on the rate of carotene destruction (n=3)

In an attempt to further elucidate results, and also aid in attempting to rank the herbs as antioxidants, the antioxidant response (AR) was calculated. Comparisons were made at the onset of the induction time in the progression of carotene oxidation.

AR's were determined and recorded as per Table 4.2 and 4.3. Independently, sage had a relatively low AR, but when combined with the other herbs, AR was higher, indicating complementary or synergistic reactions. Oregano on the other extreme, had a pro-oxidant effect on coriander and fenugreek. Both coriander and fenugreek independently had high AR's but in combination with other herbs the AR decreased. According to this method of antioxidant analysis, it has been shown that coriander and fenugreek had higher antioxidant potential, with sage and oregano showing moderate antioxidant potential.

Table 4.2: Antioxidant response of herbs by the carotene destruction (oxidation) method

Herb	Antioxidant Response (%)
Sage	5.70
Oregano	7.10
Coriander	11.30
Fenugreek (l)	12.50
Fenugreek (s)	7.30
Fenugreek (w)	12.00

(l) - leaves

(s) - stems

(w) - leaves + stems

Table 4.3: Antioxidant response of combinations of herbs ~~wrt~~by the carotene destruction method

Combination of Herbs	Antioxidant Response (%)
Sage + Oregano	9.60
Sage + Coriander	10.80
Sage + Fenugreek	9.10
Oregano + Coriander	5.30
Oregano + Fenugreek	6.30
Coriander + Fenugreek	7.20

.

4.3 ANALYSIS OF ANTIOXIDANT INDEX VIA THE RANCIMAT ~~INDUCTION PERIOD~~ METHOD

4.3.1 Analysis of Antioxidant Index of Herbs

The herbs were analyzed for potential antioxidant activity, by measuring the induction period using the Rancimat. The ability of the herbs in stabilizing sunflower oil (stripped of natural antioxidants) showed that at concentrations 20%, 40%, 60% & 80%, the antioxidant activity of sage and oregano increased with increasing concentration (Table 4.4, Figure 4.3a). The induction periods of coriander also increased with increasing concentration, but not as steadily as with sage and oregano. Fenugreek had high antioxidant activity at low concentrations, but pro-oxidant effects could be observed at higher concentrations of the herb (Table 4.4, Figure's 4.3 a & b). Again, it was observed that the fenugreek leaves are more potent as antioxidants than its stems (Table 4.4, Figure 4.3 b).

The synergistic and pro-oxidant effects of the herbs were on the stabilization of the sunflower oil was further elucidated by determining the antioxidant index value (AIV), and cumulative antioxidant index value (CAIV) calculated by using the following equations (as per Rancimat evaluation methods):

(a) Antioxidant Index Value (AIV) =

$$\frac{\text{Induction Period of oil substrate with antioxidants (herbs)}}{\text{Induction Period of oil substrate (control)}}$$

(b) Cumulative Antioxidant Index Value (CAIV) =

$$\frac{\text{Induction Period of oil substrate with mixed antioxidants (herbs)}}{\text{Induction Period of oil substrate (control)}}$$

These values are illustrated graphically – Figures 4.4 and 4.5.

The combination of the herbs indicated that this phenomena of synergism is more pronounced at a lower concentration (40%) (Table 4.5, Figure 4.4). The antioxidant potency of sage is improved upon its combination with oregano and coriander, but only at lower concentrations (Figure 4.4a). By contrast when compared with fenugreek, the induction period increased at a higher concentration. Oregano also showed synergistic behavior with coriander and fenugreek at low concentrations (Figure 4.4 b), indicating the strong synergistic properties of oregano. Coriander and fenugreek, as a combination showed lower antioxidant potency than either herb alone (Figure's 4.4 c & d). Prooxidant effects were also observed to be a characteristic of these two herbs.

Table 4.4: Induction Periods (Rancimat hrs.) of ~~herbal antioxidants~~herbs at various concentrations, in ~~stripped sunflower~~substrate oil

Concentration (%)-w/w	Induction Periods (Rancimat hours)					
	Sage	Oregano	Coriander	Fenugreek (l)	Fenugreek (s)	Fenugreek (w)
20	2.82	2.10	1.28	8.37	0.55	2.25
40	6.60	7.99	5.20	3.90	1.13	0.92
60	8.92	9.36	5.80	3.10	1.28	0.83

80	9.17	9.68	6.33	0.65	1.10	0.72

(l) - leaves

(s) - stems

(w) - leaves + stems

Table 4.5: Induction Periods (Rancimat hrs.) of combinations of ~~Herbal~~ herbs ~~Antioxidants~~ at various concentrations, in ~~stripped sunflower~~substrate oil

Concentration (%)- w/w	Induction Periods (Rancimat hours)					
	Sage + Oregano	Sage + Coriander	Sage + Fenugreek	Oregano + Coriander	Oregano + Fenugreek	Fenugreek + Coriander
40	13.10	11.47	7.52	15.35	10.85	3.50
80	3.32	4.80	12.72	3.42	0.75	6.33

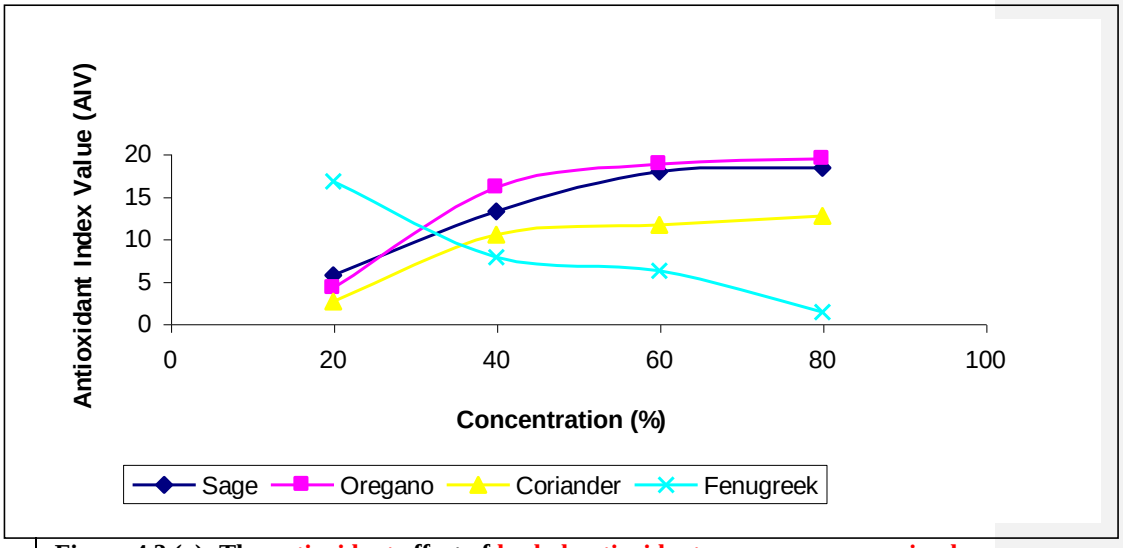


Figure 4.3 (a): The antioxidant effect of ~~herbal antioxidants~~sage, oregano, coriander and fenugreek, at various concentrations on the stabilization of sunflower oil (n=3)

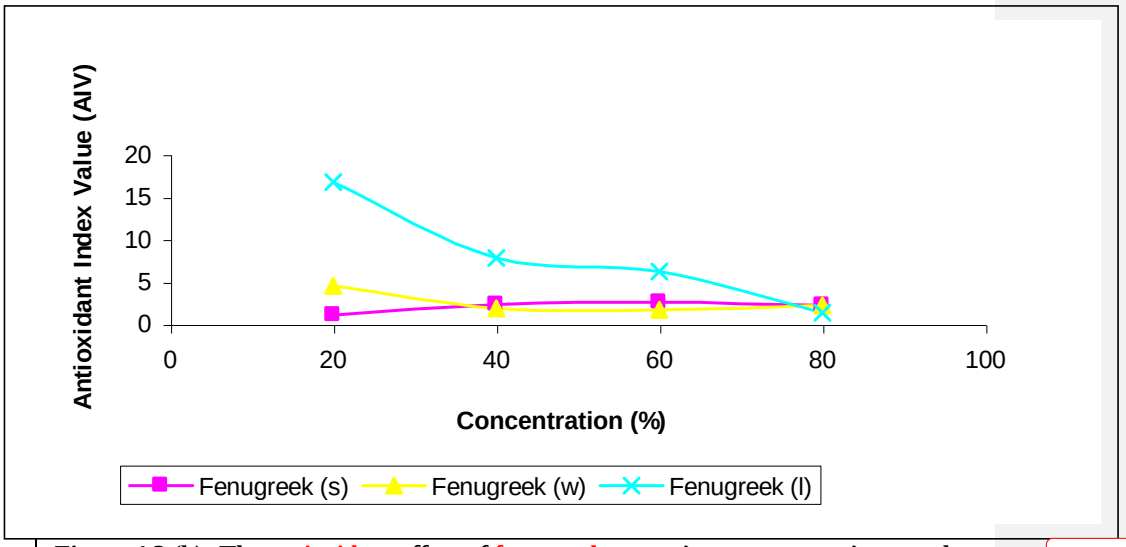


Figure 4.3 (b): The antioxidant effect of fenugreek, at various concentrations on the stabilization of sunflower oil, (l)–leaves, (s)-stems, (w)- leaves and stems (n=3)

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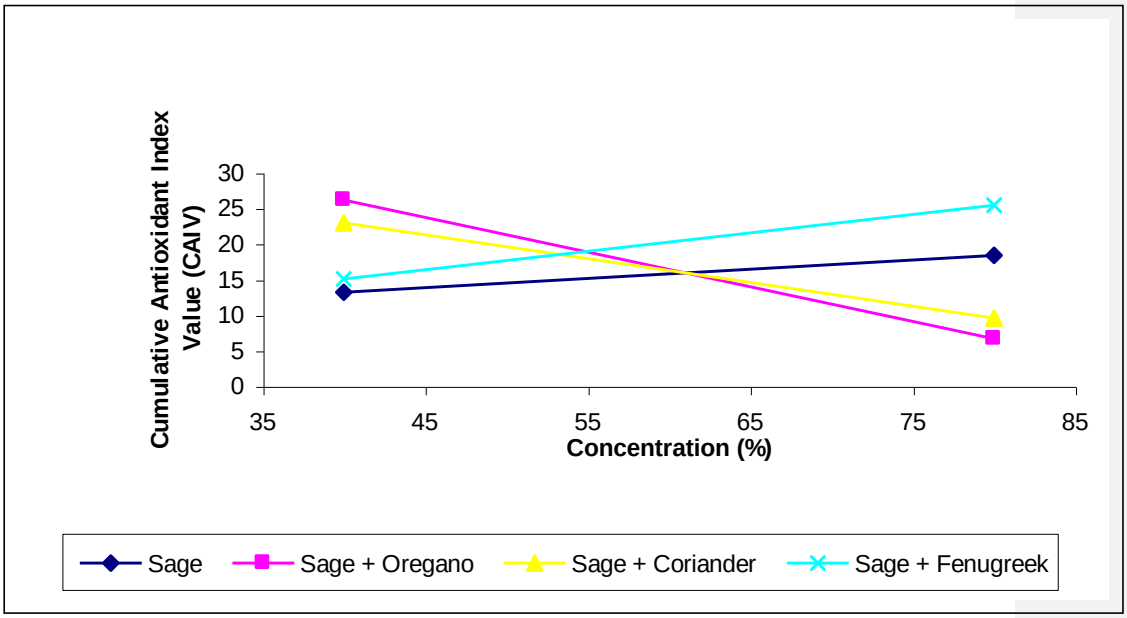
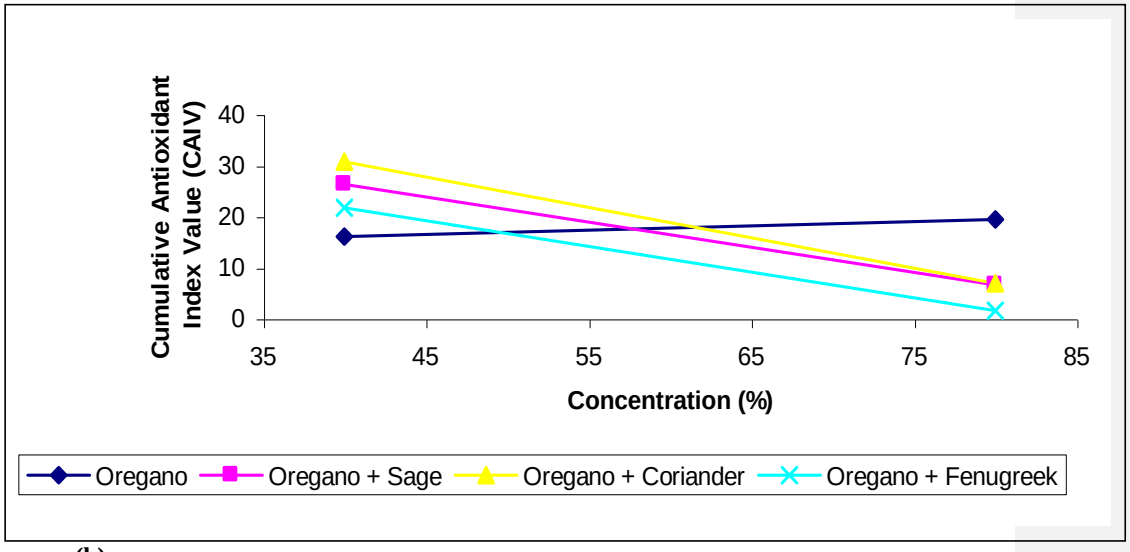
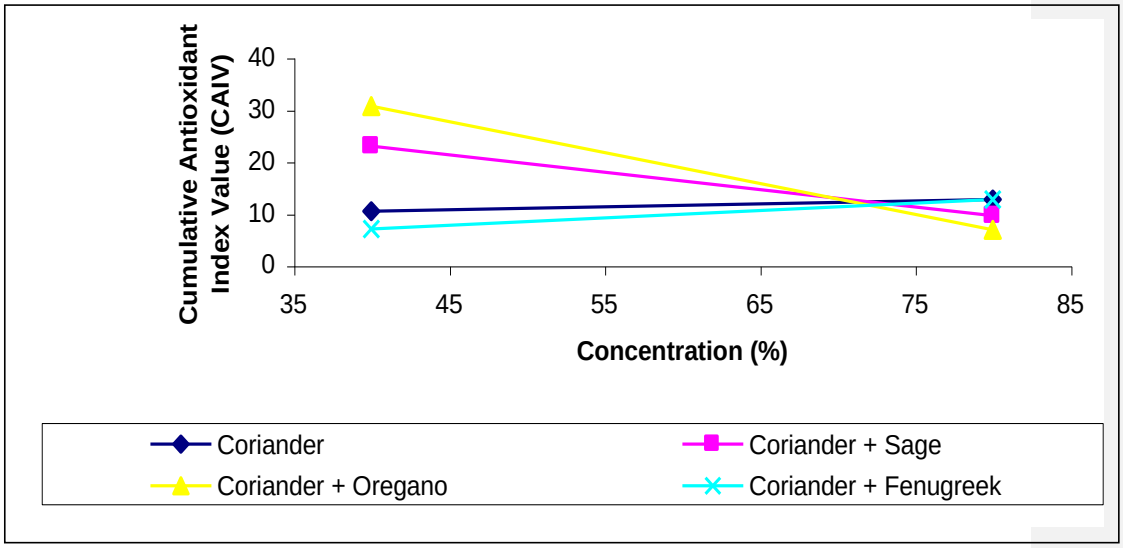

Cumulative Antioxidant Index Value (CAIV)
30
25
20
15
10
5
0
35
45
55
65
75
85
Concentration (%)
Sage
Sage + Oregano
Sage + Coriander
Sage + Fenugreek

(a)


Cumulative Antioxidant Index Value (CAIV)
40
30
20
10
0
35
45
55
65
75
85
Concentration (%)
Oregano
Oregano + Sage
Oregano + Coriander
Oregano + Fenugreek

(b)



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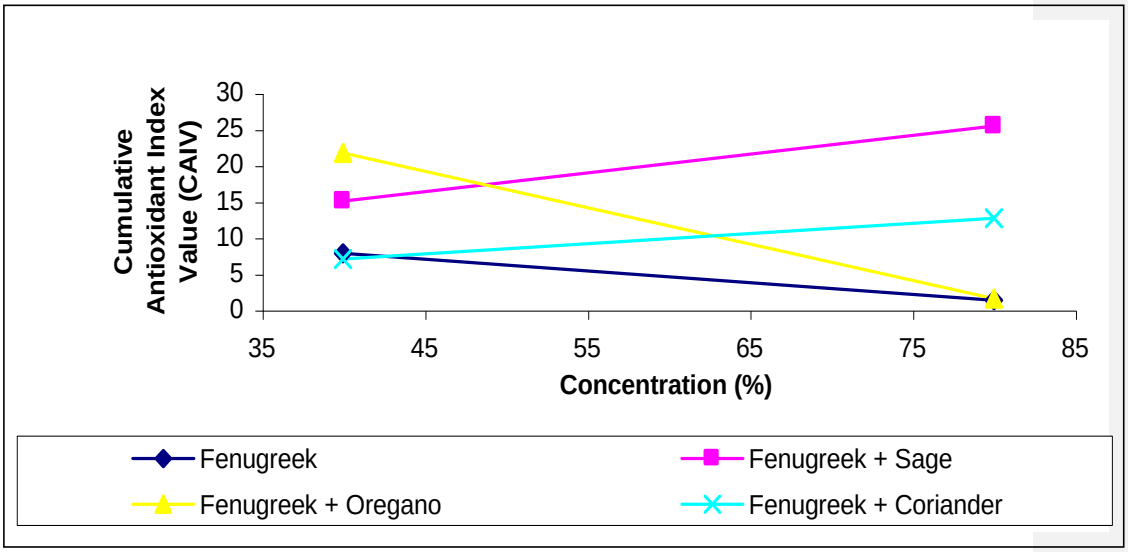

(d)

Figure 4.4: The ~~effect~~complementary and synergistic antioxidant effects of combinations of sage (a), oregano (b), coriander (c) and fenugreek (d) at various concentrations on the stabilization of sunflower oil (n=3)

4.3.2 Analysis of Antioxidant Index of Vitamins

For the control study, pure antioxidant vitamins viz. vitamin E (alpha-tocopherol), vitamin C and beta-carotene were analyzed by the Rancimat apparatus, in a similar manner to the herbs. Since pure forms of the vitamin are highly potent as antioxidants, minute amounts were used for measurement. Induction periods were recorded (Table's 4.6 and 4.7), and AIV and CAIV were calculated (Figure's 4.5 and 4.6).

Alpha-tocopherol is an efficacious antioxidant at low concentrations, but pro-oxidant at higher concentrations (> 750ppm) (Table 4.6, Figure 4.5). Vitamin C is not a very strong antioxidant on its own (Table 4.6, Figure 4.5), but was synergistic with alpha-tocopherol (Table 4.7). While it's synergistic effect with beta-carotene is less pronounced. The potency of beta-carotene is increased with increasing concentration (Table 4.6, Figure 4.5). All three vitamins in combination show strong complementary behavior (Table 4.7, Figure 4.6).

Table 4.6: Induction periods (Rancimat hrs.) of antioxidant vitamins at various concentrations, in ~~stripped sunflower~~substrate oil

Concentration (ppm)-w/w	Induction Periods (Rancimat hours)		
	Alpha-tocopherol	Vitamin C	Beta-carotene
250	4.35	0.53	0.27
500	4.55	0.48	1.00
750	0.60	0.40	1.35
1 000	0.50	0.50	4.34
1 250	0.51	0.52	4.06

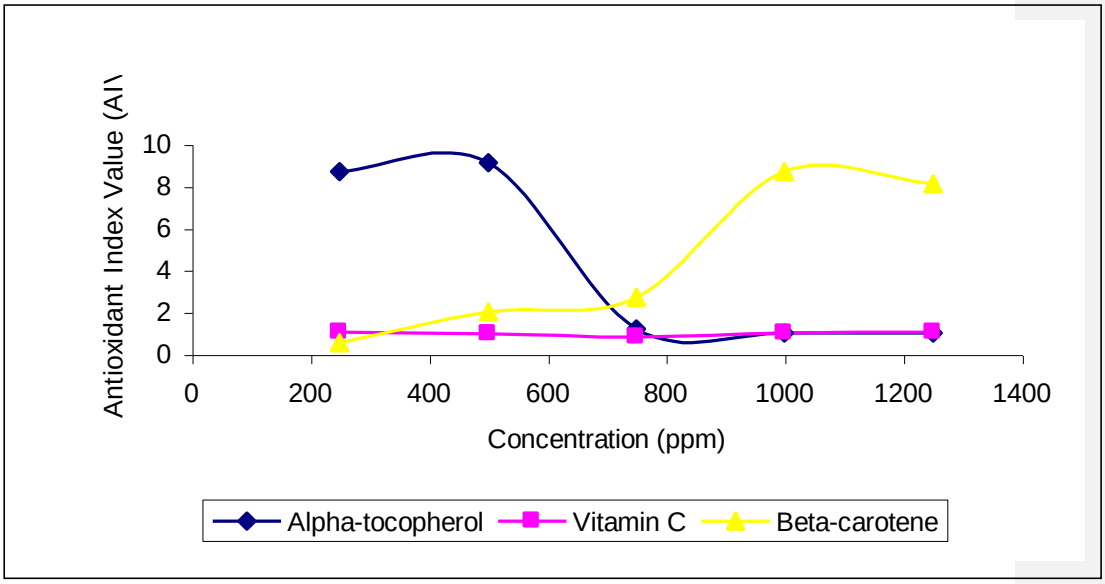

Figure 4.5: The effect of synthetic antioxidant vitamins, at various concentrations on the stabilization of sunflower oil

Table 4.7: Induction periods (Rancimat hrs.) of combinations of antioxidant vitamins at various concentrations, in ~~stripped sunflower~~substrate oil

Concentration of Vitamins (w/w)	Induction Period (Rancimat hrs.)
250ppm Alpha-tocopherol + 25mg Vitamin C	7.80
250ppm Alpha-tocopherol + 50mg Vitamin C	5.90
250ppm Alpha-tocopherol + 25mg Beta-carotene	2.17
250ppm Alpha-tocopherol + 50mg Beta-carotene	1.93
250ppm Alpha-tocopherol + 25mg Vitamin C + 25mg Beta-carotene	9.18
250ppm Alpha-tocopherol + 50mg Vitamin C + 25mg Beta-carotene	10.10

4.3.3 Antioxidant Index of herbs in a cosmetic preparation

The potency of the herbs as antioxidants in a cosmetic preparation was analyzed, by the addition of the various herbs, at various concentrations to aqueous cream. Since most cosmetics have a very definite oil component, the stability of an aqueous cream with antioxidants was assessed by the Rancimat as opposed to other types of analyses. Table's 4.8 and 4.9 summarize the results obtained from these experiments. Again, more definite analysis was carried out, by calculating the AIV and CAIV (Figure's 4.7 and 4.8), as was carried out for the herbs.

The effect of increasing antioxidant potential with increasing concentrations, was ~~obserbed~~ observed in all herbs except fenugreek (Table 4.8, Figure 4.7). Fenugreek was a very potent antioxidant at lower concentrations, for stabilizing aqueous cream, but exhibited pro-oxidant behavior at high concentrations. Coriander showed the strongest antioxidant activity for all the herbs used. Once again, stronger synergistic reactions were more pronounced at lower concentrations (Table 4.9, Figure 4.8). Sage was synergistic with oregano and coriander at low concentrations, but synergistic with fenugreek at high concentration (Figure 4.8 a). Oregano exhibited strong synergy with all herbs, at lower concentrations (Figure 4.8 b). Similar synergistic reactions were observed with coriander (Figure 4.8 c).

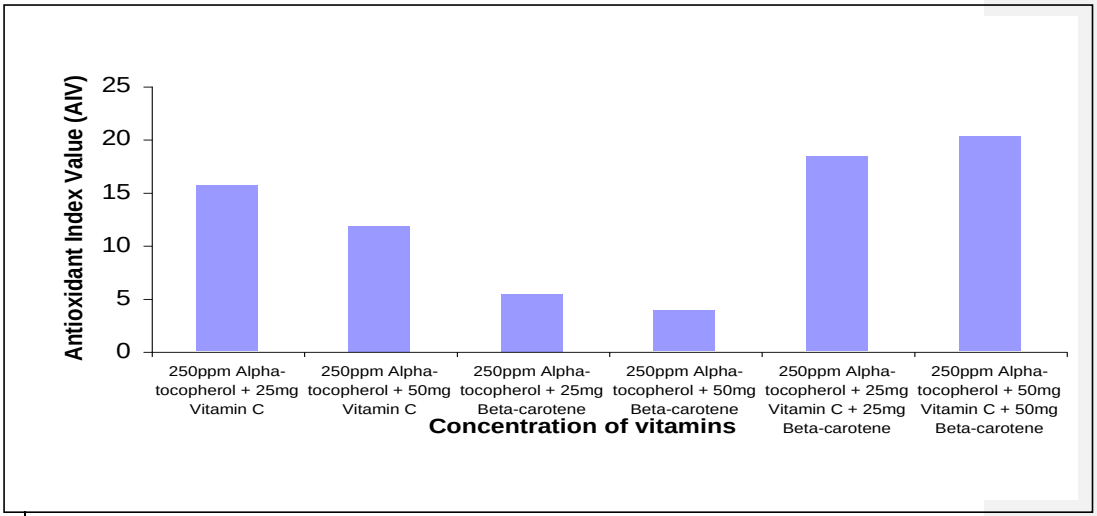

Figure 4.6: The ~~effect~~synergistic and complementary antioxidant effects of combinations of antioxidant vitamins, at various concentrations on the stabilization of sunflower oil and aqueous cream

Table 4.8: Induction periods (Rancimat hours) of ~~herbal antioxidants~~herbs at various concentrations, in ~~stripped sunflower~~substrate oil and aqueous cream

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Concentration (%)-w/w	Induction Periods (Rancimat hours)			
	Sage	Oregano	Coriander	Fenugreek
20	0.77	0.62	1.45	4.13
40	1.40	0.80	2.81	3.47
60	1.98	0.94	3.56	1.68
80	1.80	4.29	6.33	1.10

Table 4.9: Induction Periods (Rancimat hours) of combinations of herb~~al antioxidants~~ at various concentrations in ~~stripped sunflower~~substrate oil and aqueous cream

Concentration (%)-w/w	Induction Periods (Rancimat hours)					
	Sage + Oregano	Sage + Coriander	Sage + Fenugreek	Oregano + Coriander	Oregano + Fenugreek	Fenugreek + Coriander
40	3.50	9.35	3.00	3.60	5.62	3.90
60	1.32	2.20	3.40	1.33	2.48	4.06

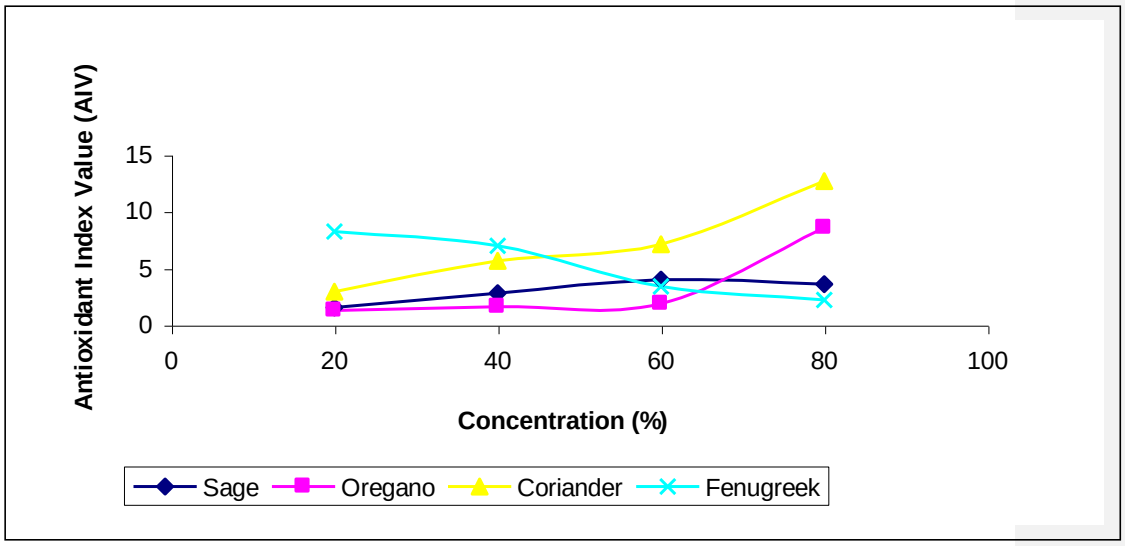
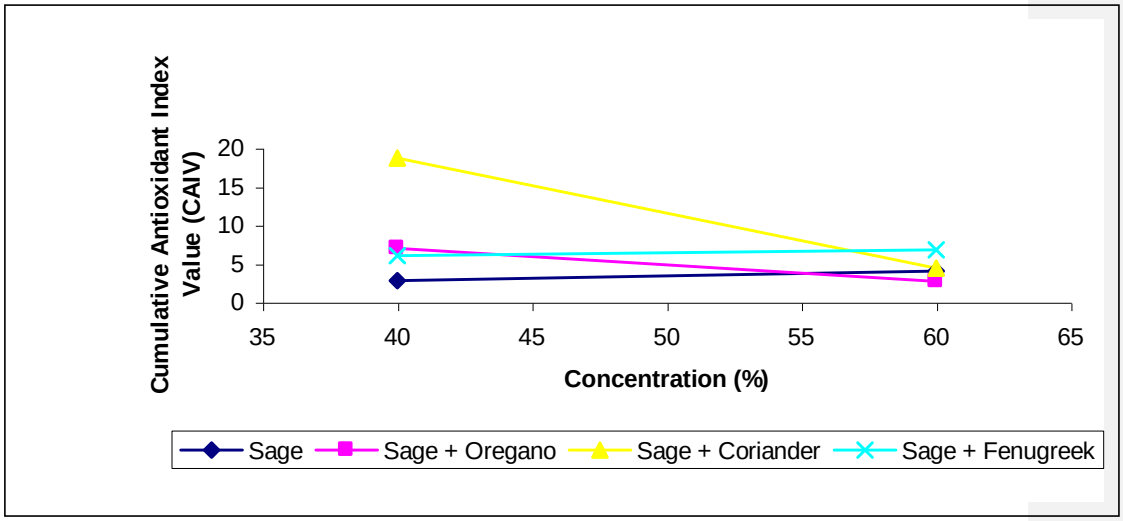
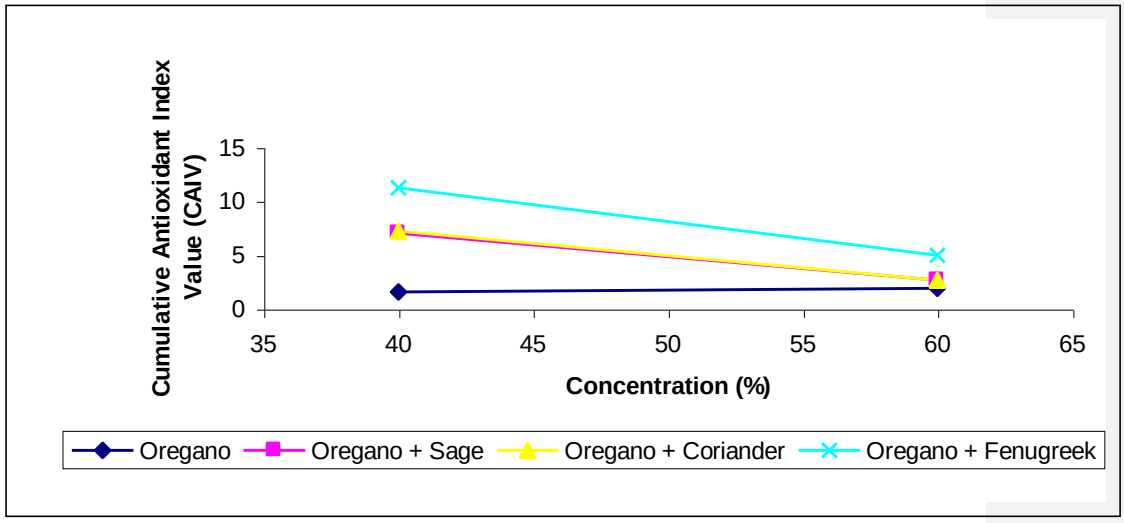


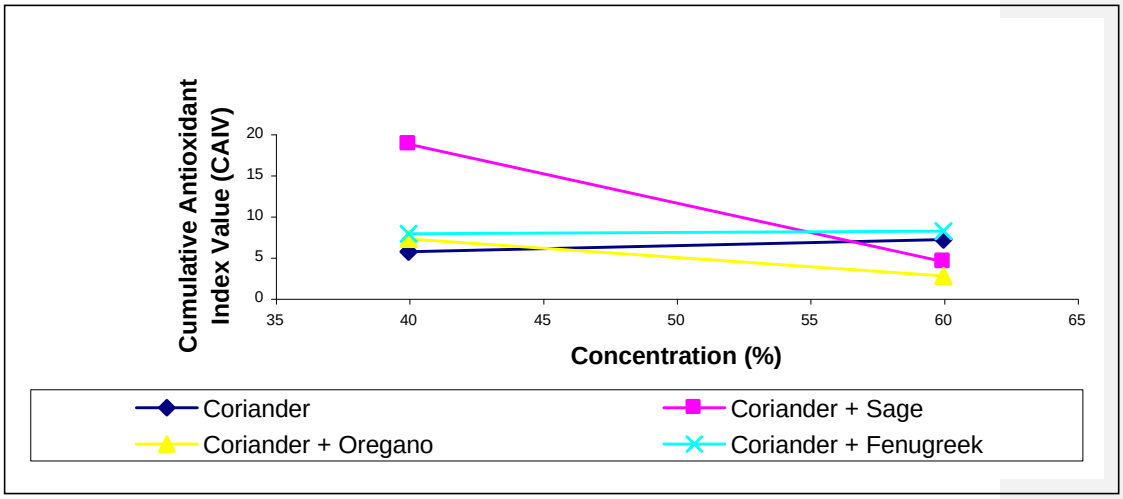
Figure 4.7: The antioxidant effect of ~~herbal antioxidants~~sage, oregano, coriander and fenugreek at various concentrations, on the stabilization of sunflower oil and aqueous cream (n=3)



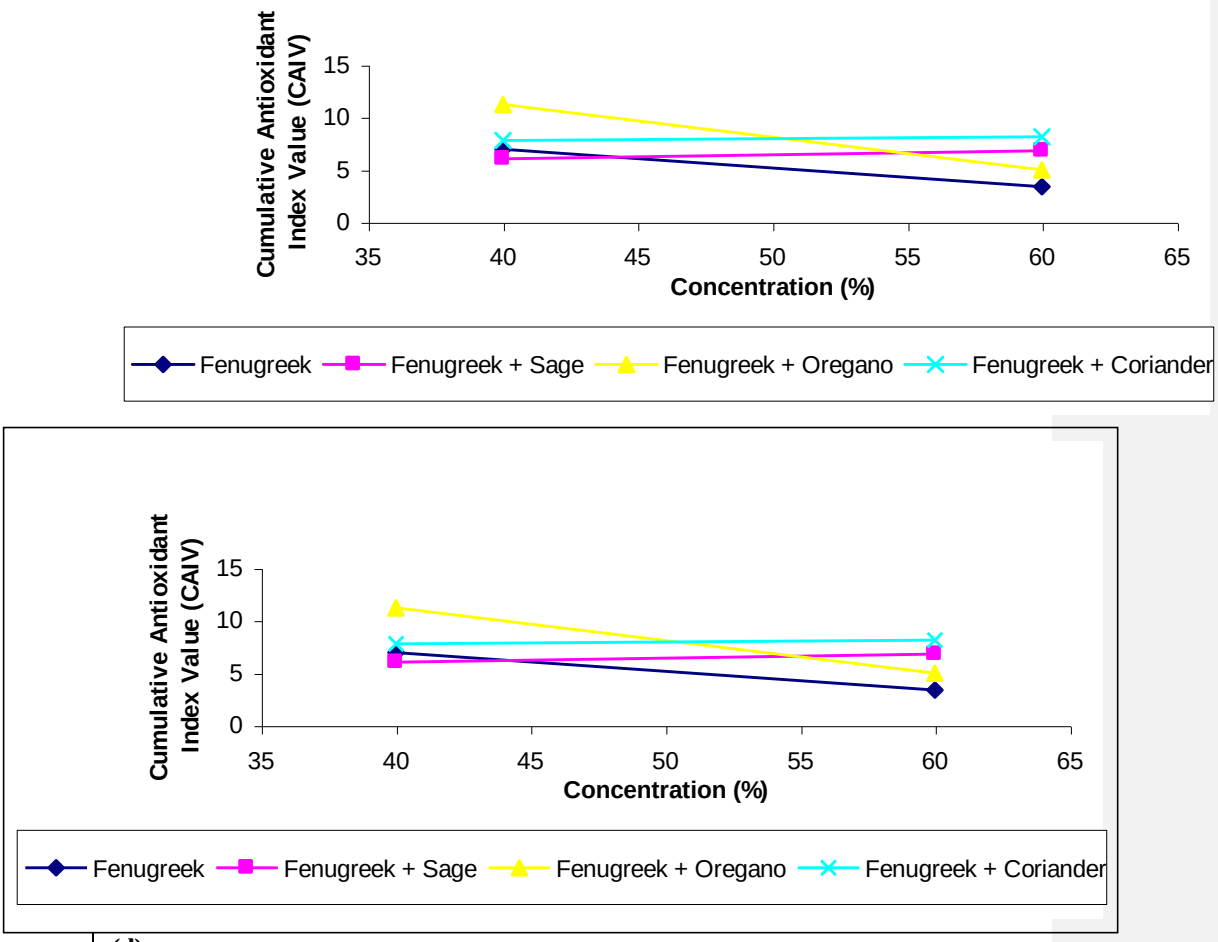
(a)


Cumulative Antioxidant Index Value (CAIV)
15
10
5
0
35
40
45
50
55
60
65
Concentration (%)
Oregano
Oregano + Sage
Oregano + Coriander
Oregano + Fenugreek

(b)


Cumulative Antioxidant Index Value (CAIV)
20
15
10
5
0
35
40
45
50
55
60
65
Concentration (%)
Coriander
Coriander + Sage
Coriander + Oregano
Coriander + Fenugreek

(c)



(d)

Figure 4.8: The ~~effect~~complementary and synergistic antioxidant effects of ~~the~~ combinations of sage (a), oregano (b), coriander (c) and fenugreek (d), at various concentrations on sunflower oil and aqueous cream (n=3)

CHAPTER 5

DISCUSSION

5.1 ANTIOXIDANT, PROOXIDANT, SYNERGISTIC AND COMPLEMENTARY INTERACTIONS OF HERBS

The first herb under investigation was sage. Sage along with rosemary, thyme and oregano belongs to the Labiatae family, which is well known for antioxidant properties (Cuvelier *et al*., 1994). Two major antioxidants present in sage, i.e. osmarinic acid and carnosic acid were identified and quantitatively determined by HPLC (Cuvelier *et al*., 1994). Djarmati and co-workers (1991) showed rosmanol-9-ethyl ether to be one of the active antioxidant components in sage, with activity greater than BHT. Further investigations have indicated that the most effective compounds in sage are carnosol, rosmarinic acid, and carnosic acid, followed by caffeic acid, rosmanol, rosmadial, genkwanin, and cirsmaritin (Cuvelier *et al.,* 1996). Sage proved to be a constant antioxidant in the reported study, exhibiting strong antioxidant activity in both preventing oxidation of beta-carotene (beta-carotene bleaching method), and in stabilizing sunflower oil (Rancimat method).

Sage retarded oil deterioration, as evidenced by retention of fatty acid profiles in palmolein oil (Jaswir *et al*., 2000). Its potency was also observed to be directly affected by its subsequent concentration, with the potency increasing as the concentration of the herb was increased (Figure 4.4 a). This correlated with the findings of Burkow *et al.* (1995) who reported that based on the Rancimat measurement, increased antioxidant induction period for sage with increasing concentration. Moreover, in preserving alpha-tocopherol in sunflower oil, the preservation of alpha-tocopherol was directly related to the concentration of sage extract (Beddows *et al*., 2000).Sage also complemented the other herbs used in the study, by increasing their antioxidant potential (Tables 5.1 & 5.2). This effect was further enhanced at a lower

Table 5.1.: Cross-referenced results of Tables 4.2 & 4.3 depicting Antioxidant response values calculated by Carotene Oxidation Method

Antioxidant Response (%)	**Sage**	**Oregano**	**Coriander**	**Fenugreek (l)**	**Fenugreek (s)**	**Fenugreek (w)**
Sage	5.70	9.60	10.80	9.10		
Oregano		7.10	5.30	6.30		
Coriander			11.30	7.20		
Fenugreek (l)				12.50		
Fenugreek (s)					7.30	12.0
Fenugreek (w)						

(l)- leaves, (s)- stems, (w)- leaves + stems

Table 5.2.: Cross-referenced results of Tables 4.4 & 4.5 depicting Induction Periods of herbs in substrate oil, calculated by Rancimat Method

Induction Periods (Rancimat hours)		Sage		Oregano		Coriander		Fenugreek (l)		Fenugreek (s)		Fenugreek (w)	
		40%	**80%**	**40%**	**80%**	**40%**	**80%**	**40%**	**80%**	**40%**	**80%**	**40%**	**80%**
Sage	**40%**	6.0		13.10		11.47		7.52					
	80%		9.1		8.32		4.80		12.72				
Oregano	**40%**			7.99		15.35		10.85					
	80%				9.68		3.42		0.75				
Coriander	**40%**					5.20		3.50					
	80%						6.33		6.30				
Fenugreek (l)	**40%**							3.90					
	80%								0.65				
Fenugreek (s)	**40%**									1.30			
	80%										1.10		
Fenugreek (w)	**40%**											0.92	
	80%												0.72

(l)- leaves, (s)- stems, (w)- leaves + stems

concentration (40%) with oregano and coriander, but at higher concentration with fenugreek (80%). It was therefore prooxidant in its reaction with fenugreek, at a lower concentration. Sage can therefore be used alone, or in combination with the other herbs but at very specific concentrations. More quantitative studies will be necessary in order to establish specific concentrations to be used. This should therefore be the focus of further related studies.

Oregano is also a potent antioxidant, independently or in combination with the other herbs under investigation. It was very effective in preventing the destruction of carotene, both independently as well as synergistically with sage, coriander and fenugreek. (Table 5.1). The main antioxidant factors isolated from oregano are flavonoids (Vekiari *et al*., 1993). Subsequent chromatographic and spectrophotometric analyses have demonstrated the presence of the flavone, apigenin, the flavonone, eridictyol and the dihydroflavonols, dihydrokaemferol and dihydroquercetin (Vekiari *et al*., 1993). Oregano was shown to contain various antioxidant pyrocatechol derivatives, including 4-(3,4-dihydroxy benzoyloxymethyl)-phenyl-β-D-glucopyranoside (Pokorny, 1991). In addition, carvacrol and thymol have been isolated from the essential oil of oregano (Madsen and Bertelsen, 1995). Its effect as an antioxidant was significantly more pronounced in complementing sage, more than coriander and fenugreek (Tables 5.1 & 5.2). In relation to its antioxidant activity being influenced by its concentration, an almost direct relationship could be observed (Figure 4.3 a). When considering the use of oregano as an antioxidant, this property should be taken into consideration.

Oregano was also a strong synergist in complementing the antioxidant activity of the herbs in stabilizing sunflower oil (Figure 4.4 b, Table 5.2). This phenomenon was however highlighted at lower concentrations of the herb. Hence, if oregano is to be used as a synergist, it should be noted that its effect is heightened at a lower concentration. When antioxidant activities of some plant extracts of the family Labiatae were tested in lard stored at room temperature, oregano was found to be the most effective followed by thyme, dittany, marjoram and lavender extracts in decreasing order (Economou *et al*., 1991).

Coriander proved to be one of the superior herbs under investigation in preventing the oxidation of beta-carotene (Table 4.2). However, its effect as an antioxidant in stabilizing sunflower was less striking, than that of the other herbs (Table 4.4). More interestingly. in a recent study, when herb extracts were used to preserve alpha-tocopherol in sunflower oil during heating, coriander was a pro-oxidant (Beddows *et al*., 2000), as opposed to the antioxidants: rosemary, sage, oregano and cumin extracts.

Coriander's activity as an antioxidant is however improved with increasing concentration of the herb (Table 4.4). This should be an important consideration in the use of coriander as an antioxidant. In preventing the oxidation of beta-carotene, coriander complemented the antioxidant potency of sage, but exhibited prooxidant mechanisms with oregano and fenugreek (Figure 4.2 c, Table 5.1). In stabilizing sunflower oil, it proved to be a strong synergist with sage and oregano, and together with fenugreek (Figure 4.4 c, Table 5.2). These results indicated a stronger tendency for coriander to be used independently as an antioxidant, rather than as a synergist.

Studies investigating the antioxidant activity of fenugreek are lacking in the literature, hence indicating the potential for some breakthrough research findings. However, literature indicating its use as a medicinal herb is very vast. Fenugreek is used in India in its indigenous system of medicine to treat a number of diseases: it is vegetable insulin, retards retinopathy in rats and is a scavenger of superoxide and hydroxyl radicals (Gupta, 1998). This limited research ~~promted~~prompted the necessity to analyze the different components of the plant for potential antioxidant activity, in order to establish the source of potential antioxidant components. The leaves proved to be the centre of antioxidant activity, housing the antioxidant components. This was concluded from the analyses through the beta-carotene bleaching experiments, and in stabilizing sunflower oil by the Rancimat method. Possible pro-oxidant components are proposed to be present in the stems of the fenugreek plant, and thus may have played a role in lowering the overall antioxidant activity of the entire plant.

Fenugreek leaves were the most effective herbs as antioxidants in delaying the onset of oxidation in beta-carotene, as concluded from the beta-carotene bleaching method (Table 4.2). Fenugreek leaves were also a synergist, in complementing the antioxidant activity of the other herbs (Table 5.1). Fenugreek, unlike the other herbs under investigation, is a pro-oxidant at higher concentrations (Table 4.4). This aspect of fenugreek could be the motivation for further exciting ~~researchr~~research. This is also a very important factor to be considered in the use of fenugreek as an antioxidant. As with the other herbs under investigation, it has become quite evident that concentration of antioxidants is a very significant factor in their use. Fenugreek was also synergistic with sage and oregano, at lower concentrations, in stabilizing sunflower oil and prooxidant at high concentrations with coriander (Table 5.2).

Strong antioxidant function was recognized in all herbs under study. Sage, oregano, and coriander's efficacy as antioxidants was increased with increasing concentration, but fenugreek is a prooxidant at higher concentrations. Further research could aim to establish the optimum level of concentrations of the herbs to be used as antioxidants. In terms of synergistic reactions, sage and oregano were the best synergists overall.

5.2 CUMULATIVE ANTIOXIDANT ACTVITY IN COSMETIC FORMULATION-AQUEOUS CREAM

Similar effects were observed for the herbs in stabilizing aqueous cream, as in delaying oxidation of beta-carotene (beta-carotene bleaching) and in stabilizing sunflower oil. Again, a direct relationship was observed between the concentration of herbs and antioxidant action (Figure 4.7) for sage, oregano and coriander. The prooxidant effect of fenugreek at higher concentrations was also observed. It can therefore be concluded that the mechanisms by which antioxidants stabilize food products also operate in cosmetic products. The possibility of the use of the herbs in combinations, to further optimize antioxidant activity was assessed (Table 4.9). As previously discussed (Chapter 5.1) sage and oregano are excellent synergists, and in this case complementing the other herbs in

Table 5.3.: Cross-referenced results of Tables 4.8 & 4.9 depicting Induction Periods of herbs in substrate oil and aqueous cream, calculated by Rancimat Method

Induction Periods (Rancimat hours)		Sage		Oregano		Coriander		Fenugreek (l)	
		40%	60%	40%	60%	40%	60%	40%	60%
Sage	40%	1.40		3.50		9.35		3.0	
	60%		198		1.32		2.20		3.40
Oregano	40%			0.80				5.62	
	60%				0.94				2.48
Coriander	40%			3.60		2.81		3.90	
	60%				1.33		3.56		4.06
Fenugreek (l)	40%							3.47	
	60%								1.68

stabilizing the cosmetic formulation (Figure 5.3). It was again observed, that these synergistic reactions are more pronounced at lower concentrations, with prooxidant reactions occurring at higher concentrations. Coriander exhibited moderate synergistic behavior (Figure 4.8 c. Table 5.3). Fenugreek was more effective in its use as an antioxidant independently, rather than a synergist (Figure 4.8 d).

The antioxidant vitamins have long played a role in cosmetics. Thus, natural sources of these vitamins could prove beneficial. Vitamin C is a powerful antioxidant and potential wrinkle-reducing agent (Knowlton, 2001). Vitamin C when topically applied in the correct form, functions as a biologic co-factor and free radical scavenger, alleviating the effects of natural vitamin C loss from the body to a large degree (Knowlton, 2001). Vitamin C and its derivatives are actives that play an important role in the skin-lightening process by competing with melanin precursors for the oxidative stress available to the system (Tew, 2000). Vitamin E prevents skin damage by free radicals and protects the skin against UV damage (Tew, 2002). Beta-carotene by binding in a competitive way to the cellular receptors of vitamin A, well known for its properties of stimulation of melanocyte activity, acts by temporarily 'freezing' melanogenesis (Tew, 2002). The vast properties of these vitamins as antioxidants are consequently unlimited, thus showing immense potential in the cosmetic industry. Natural sources of these vitamins will prove to be even more acceptable to the consumer, thus providing a niche for suitable research, as in the reported study.

5.3 EFFECT OF CONCENTRATION OF ANTIOXIDANT VITAMINS ON ANTIOXIDANT ACTVITY IN HERBS

Plants synthesize the well known antioxidants tocopherols, ascorbic acid and carotenoids (Vinson *et al.*, 1998). It should be noted at the onset that the herbs under investigation contain many other substances that have antioxidant functions besides these antioxidant vitamins. The antioxidant potency of vitamins, with special reference to vitamin's C and E and beta-carotene has been well documented in the scientific literature. One of the objectives of the reported study was to determine to what extent the concentration of

antioxidant vitamins in each herb was responsible for its overall antioxidant potential of that herb. In order to verify this, a control study was conducted, in which the induction periods of the pure antioxidant vitamins were measured via the Rancimat method.

One of the deductions made earlier, was the prooxidant tendencies of fenugreek at higher concentrations. It was also determined that fenugreek had a high concentration of vitamin C (650.00 mg/kg), compared to the other herbs. It is even more interesting to note the prooxidant course of action of the pure vitamin C at high concentrations (Table 4.6). Aruoma (1996b) reported that vitamin C can act as a prooxidant, which correlates to the study. In studying the interacting effects of ascorbic acid and metal ions on carotene oxidation in aqueous carotene-linoleate solution, it was found that ascorbic acid at concentrations up to 10^{-3} M was a prooxidant (Kanner and Mendez, 1977). A clear correlation was therefore concluded: the concentration of antioxidant vitamins has an indirect effect on the overall antioxidant capacity of the herb in question.

Coriander was the herb with the highest concentration of all antioxidant vitamins (Table 4.1). It was also noted that a combination of all three antioxidant vitamins was effective in stabilizing sunflower oil (Table 4.7). This maybe indicative of the mechanism by which coriander stabilized sunflower oil. The high concentrations of the antioxidant vitamins in coriander could have contributed to its overall performance as an antioxidant. In a 10% oil-in-water emulsion, the prooxidant effect of beta-carotene was inhibited only in combination with antioxidants such as α- and γ-tocopherols (Heinonen, 1997). Heinonen (1997) reported that the combination of beta-carotene and alpha-tocopherol was significantly better in retarding oxidation than alpha-tocopherol alone, in oil-in-water emulsions of rapeseed oil triacylglycerols. Sage and oregano had very similar concentrations of the antioxidant vitamins. This is probably the reason behind the similar antioxidant effects seen with these two herbs. It has also been established that there exists a close botanical relationship between sage and rosemary (Madsen and Bertelsen, 1995), seeing that they contain the same antioxidant compounds: carnosol and carnosic acid.

Furthermore, it was observed that the antioxidant vitamins concentrations in the herbs may have had an effect on the synergistic and prooxidant reactions observed. Fenugreek and coriander were not very good synergists (Tables 5.1 & 5.2), as concluded in earlier discussion. These herbs also contained high concentrations of the vitamins. As reported in Table 4.6, alpha-tocopherol and vitamin C are prooxidants at high concentrations. Again a distinct correlation can be seen, the high concentration of these vitamins in the herbs, when combined is much higher therefore tending towards prooxidant behavior. Pokorny (1991) reported that at high concentrations and in the presence of traces of iron and copper salts, tocopherols may act as prooxidants; satisfactory antioxidant activity is usually achieved only when they are used as ascorbic acid, citric acid and some amino acids, or with various chelating agents.

According to Kitts (1997) many workers using different model food systems have reported the prooxidant property of vitamin E. The potential for prooxidant activity occurs at high concentrations of vitamin E during the autoxidation of polyunsaturated fatty acids. The mechanism for this response has been attributed to the affinity of vitamin E at high concentrations to trap peroxyl radicals by hydrogen-atom abstraction and chain-transfer reaction, which alters the stereochemistry of the hydroperoxides formed (Kitts, 1997). Huang *et al.* (1994) also found in bulk corn oil, alpha-tocopherol had either antioxidant or prooxidant activity depending on concentration, oxidation time, the method of determining oxidation, and physical state. And, on the basis of hydroperoxide formation, alpha-tocopherol had an antioxidant effect at 100 ppm in tocopherol-stripped corn oil and a prooxidant effect at higher concentrations at the early stage of oxidation. In addition, ~~Koskas~~Kushi *et al.* (1984) reported that an increase in the concentration of alpha-tocopherol caused a conversion of its antioxidant activity to prooxidant activity. This phenomenon resulted in an increase of hydroperoxide formation. Moreover, alpha-tocopherol at high concentration exhibited a prooxidant effect during autoxidation of linolenic and arachidonic acids (Husain *et al.*, 1987).

Herbs with a moderate concentration of the vitamins viz. sage and oregano were very good synergists because when combined with the other herbs, the overall concentrations

of antioxidant vitamins would have been adequate to obtain maximum antioxidant potential. It is unlikely that vitamins with antioxidant activity only act independently; rather vitamin C, alpha-tocopherol and beta-carotene may work in cohort under certain conditions to produce optimal protection against oxidative stress (Kitts, 1997). In fact, the overall antioxidant capacity of a complex mixture corresponds to the sum of each antioxidant concentration times its rate constant for the interaction with a peroxyl radical (Tubaro *et al*., 1996).

5.4 BETA-CAROTENE BLEACHING METHOD VERSUS RANCIMAT METHOD

The methodology used to evaluate natural antioxidants must be carefully interpreted depending on the conditions of oxidation and the analytical method used to determine the extent and endpoint of oxidation (Frankel, 1993). The literature on stability evaluations of food lipids is extensive. However, the published data comparing the effectiveness of various antioxidants is often difficult to interpret because of questionable methodology; particularly the choice of method utilizes inappropriate oxidation conditions (Frankel, 1993). Natural antioxidants have been especially difficult to evaluate because of the use of crude extracts. The occurrence of complex interfacial phenomena in oils and food emulsions has further compounded the analytical problems (Frankel, 1993). Given the current interest in the use of natural antioxidants in foods and the development of new vegetable oils and blends, it is appropriate to re-evaluate the methods currently being used to determine the oxidative stability of food lipids and edible oils, and to test the effectiveness of antioxidants (Frankel, 1993).

As previously reported, different methods to analyze antioxidants can produce conflicting results. This was the case in the reported study. It was found that the two methods produced conflicting data, in terms of ranking the herbs as antioxidants. The beta-carotene method was developed as a rapid, sensitive method for ranking antioxidant activity based upon minimizing beta-carotene loss in the coupled oxidation of linoleic acid and beta-carotene using an emulsified, aqueous system (Marco, 1968). According to

the beta-carotene bleaching method the herbs were ranked as follows for ~~antixoxidant~~antioxidant response: fenugreek > coriander > oregano > sage (Table 5.1). Using the Rancimat method herbs were ranked as follows: oregano > sage > coriander > fenugreek (Table 5.2).

Many possible explanations can be offered to explain these differences. The order of activity and ranking of different antioxidants depend on whether they are tested at high or low temperatures (Frankel, 1993). An Arrhenius plot of log (overall reaction constant) versus 1/T shows that the antioxidant effectiveness increases as T (the temperature) decreases (Frankel, 1993). The overall protection predicted at high temperature for an antioxidant will usually be less than that found at lower temperatures (Frankel, 1993). The limitations of high-temperature stability tests include the following: the rate of oxygen becomes dependent on oxygen concentration, because the solubility of oxygen decreases to elevated temperatures; oxidation occurs rapidly and results in drastic changes in oxygen availability; the IP occurs at an oxidation level that is too high and beyond the point at which rancid flavours are detected; side reactions such as polymerization and cyclization become important and may not be relevant to normal storage temperature; analyses of oxidation under these conditions are of questionable value; phenolic antioxidants in natural extracts decompose at elevated temperature (Frankel 1993). This could be case of the Rancimat.

The instability of vitamin C when exposed to environmental conditions such as heat, high oxygen tension or low pH results in the formation of oxidation or degradation products (e.g. dehydroascorbic acid, diketogulonic acid and 2-hydroxyfurfural), as well as the loss of antioxidant activity (Kitts, 1997). This could be the case for fenugreek and coriander, which have high concentrations of vitamin C; instability of vitamin C at the high temperature used in the Rancimat could have attributed to loss of some antioxidant activity. Hasenhuetti and Wan (1992) reported that, at higher temperature, stability of olive oil towards oxidation was much lower than in the Rancimat. This is ~~a~~ another possible explanation for the differences seen in the two methods. Burkow *et al*. (1995) reported from their investigation on the evaluation of antioxidants for cod liver oil by

chemilumescence and the Rancimat: ranking of antioxidants varied considerably depending on the method used, and increasing the temperature seemed to decrease the usefulness of the method. It is therefore probable that any one of these reasonings could be applicable in the reported study, for differences in results from the two methods. Although the high temperature used in the Rancimat seems to be the probable cause, it should not contravene the advantages of this apparatus. The Rancimat helped to provide information fairly quickly and showed the relationship between the delay in rancidity and the preservation effect of the herbs. A future recommendation would be the use of a lower temperature. Beddows and colleagues (2000) recommended a temperature of 85^0C for the evaluation of herbs and spices in the Rancimat.

Frankel (1993) because of the variation in results due to the different methods concluded that "there is a need for standardization of methods to determine the oxidative stability of different food lipid systems and different antioxidant systems", he recommends a testing protocol, in which different storage temperatures and different types of fats are used, and where the oxidation progress is measured by more than one method. In ~~an~~ a contrasting view, Meyer (1994) proposed the development and use of a wider range of standard model systems for testing new antioxidants, and complexity, where the highest, most complex level closely resembles the selected product requiring oxidative protection. These complex models could replace the use of standardized, 'ideal' accelerated test systems for evaluating antioxidant efficacy.

CHAPTER 6

CONCLUSIONS

The reported study was conclusive in finding all the herbs, under investigation to contain functional components, which contributed to antioxidant activity. The methods used to analyze antioxidant activity, although producing varying results~~,~~ were sufficient in providing data to assess the herbs for antioxidant activity. Several deductions were made on the antioxidant potential of each herb, as well as their combination in substrates consisting of sunflower oil and a base cosmetic preparation.

Sage, oregano and coriander are herbs that have been researched previously for their antioxidant functions. In this study, these herbs were satisfactory ~~antixoxidants~~antioxidants, independently and in combinations. Sage and oregano were excellent synergists, whereas coriander showed moderate synergistic behaviour. All three herbs showed a direct relationship between herb concentration and antioxidant activity. Fenugreek, on the other hand was a prooxidant, and greatly so at higher concentrations. This however does not discard its function as an excellent antioxidant in the reported study.

It was therefore deduced that when using antioxidants in combinations, it should first assessed which antioxidants are synergistic or pro-oxidant. Overall the herbs were ranked as follows, according to the two methods of analysis: fenugreek > coriander > oregano > sage (Beta-carotene bleaching method), oregano > sage > coriander > fenugreek (Rancimat method).

It was also concluded that the mechanisms by which antioxidants react in a food product would be similar to that in a cosmetic product. This was deduced on the basis of how the herbs and combinations thereof reacted similarly in both the oil substrate and the cosmetic formulation. The control study, in which antioxidant vitamins were assessed, was very useful in aiding understanding of the various interactions that were taking place

amongst the herbal antioxidants. For example, the high vitamin C content of fenugreek could have been been attributed to its prooxidant tendencies, as vitamin C is known to be aproxidant at high concentrations.

The reported study was one of many in investigating the exciting and innovative topic of antioxidants. Antioxidants are proving to be an indispensable component of many disciplines, from food to medicine. More research is required for the identification and isolation of these functional antioxidant components, and as reported the natural route is very good source. The benefits of utilizing a natural source for antioxidants will be widely welcomed by the beauty market worldwide. Natural antioxidants are even more attractive because natural sources are the safest of ingredients and of course antioxidants are the key to taking off years in appearance. Many women and some men will be most keen to try products containing such key ingredients. The role of antioxidants in food products are not often made aware to the end consumer, but the manufacturers will surely want to increase the shelf life of their products, preventing rancidity and spoilage at the same instant. The need for antioxidants in medicine is even more a necessity and the massive research into this area is indicative of the far reaching consequences it can have if not already on the treatment of serious ailments and diseases. The key to unlocking the mechanisms and functional components of various natural sources of antioxidants lies in research and the the reported research project has contributed to this vast abundance of investigative research into natural antioxidants.

CHAPTER 7
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APPENDICES

APPENDIX A

Absorbencies of solutions containing beta-carotene and herbs at 470nm

Time (min.)	Control	Sage	Oregano	Coriander	Fenugreek (whole)	Fenugreek (leaves)	Fenugreek (stems)
0	0.420	0.470	0.428	0.438	0.468	0.458	0.462
15	0.420	0.443	0.427	0.420	0.447	0.448	0.461
30	0.398	0.413	0.404	0.402	0.399	0.429	0.428
45	0.345	0.366	0.384	0.402	0.394	0.361	0.416
60	0.323	0.358	0.360	0.399	0.393	0.349	0.393
75	0.302	0.334	0.340	0.377	0.378	0.344	0.389
90	0.257	0.314	0.328	0.370	0.377	0.330	0.382
115	0.241	0.287	0.325	0.325	0.364	0.321	0.374
130	0.199	0.278	0.316	0.325	0.360	0.308	0.350
145	0.180	0.264	0.313	0.296	0.321	0.306	0.290
160	0.165	0.186	0.217	0.263	0.251	0.283	0.270

APPENDIX B

Absorbencies of solutions containing beta-carotene and combinations of herbs at 470nm

Time (min.)	Control	Sage + Oregano	Oregano + Coriander	Oregano + Fenugreek	Sage + Fenugreek	Sage + Coriander	Fenugreek + Coriander
0	0.441	0.450	0.431	0.441	0.423	0.469	0.407
15	0.415	0.428	0.420	0.433	0.423	0.413	0.405
30	0.366	0.413	0.387	0.391	0.404	0.412	0.401
45	0.361	0.377	0.357	0.374	0.395	0.402	0.349
60	0.359	0.374	0.338	0.359	0.387	0.396	0.333
75	0.357	0.357	0.328	0.337	0.378	0.384	0.330
90	0.320	0.353	0.310	0.320	0.348	0.365	0.329
115	0.270	0.340	0.302	0.318	0.318	0.311	0.321
130	0.245	0.337	0.296	0.304	0.317	0.319	0.305
145	0.283	0.310	0.261	0.297	0.296	0.317	0.282
160	0.191	0.283	0.240	0.290	0.285	0.284	0.275

APPENDIX C

1. Meta-phosphoric acid (MPA)

15 g of meta-phosphoric acid was dissolved in a mixture of 40 ml glacial acetic acid and 200 ml of distilled water. The mixture was diluted to 500 ml with distilled water, in a volumetric flask and thereafter filtered with Whatman no.4 filter paper. This reagent was stable for 7-10 days, provided it was stored in the refrigerator.

2. Indicator

50 mg phenol-indo-2:6-dichloro-phenol was dissolved in 150 ml hot water, containing 42 mg of sodium hydrogen carbonate. This mixture was cooled and diluted to 200 ml with distilled water, following which it was filtered with Whatman no. 4 filter paper, and then stored in the refrigerator in amber glass bottles. This reagent can be stable for seven days.

3. Vitamin C Standard

100 mg of ascorbic acid was accurately weighed and dissolved in approximately 10-15 ml M.P.A solution. This solution was then diluted to 100 ml with M.P.A solution. This solution was only stable for a day, and therefore had to be made fresh.

APPENDIX D

1. Substrate oil: Kieselghur and activated charcoal treatment to remove natural antioxidants from sunflower oil

Twenty grams of a sunflower oil sample was diluted with 40 ml diethyl ether. The mixture was treated with either 5 g kieselghur or with 2 g of a 1:1 mixture of kieselghur and activated charcoal. The mixture was thoroughly mixed in a 2.5 l amber glass jar. The treated mixture was filtered with Whatman no. 4 filter paper, placed under vacuum and the ethereal filtrate evaporated to the original oil level. For the double treatment a further 2 g of the kieselghur/activated charcoal mixture was added and the above discussed process repeated. The treated oil samples were stored at -16^0C and used as substrate oil in the Rancimat measurements.

APPENDIX E

1. Aqueous cream formulation

Forty-eight milliliters water, 1.16 g sodium hydroxide and 33.24 g glycerine were weighed into a beaker. The mixture was heated to 75^0C (mixture A). 16.62 g stearic acid was measured into a second beaker and heated to 75^0C (mixture B). Mixture A was allowed to cool to room temperature preceding the addition of 6.64 g aloe vera oil. Mixture A was added to mixture B and mixed thoroughly then heated to 75 ^{0}C. The mixture was then cooled to room temperature and 1.66 g Classique (Dragoco) fragrance was added. The mixture was thoroughly stirred until all lumps were broken. The contents were then transferred and stored in plastic bottles until further use.