Extraction, preservation, and utilization of natural colour from marigold (*Tagetus erecta* L.)

By

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(2007-12-101)

THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Extraction, preservation, and utilization of natural colour from marigold (*Tagetus erecta* L.)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that the thesis entitled **"Extraction, preservation, and utilization of natural colour from marigold (*Tagetus erecta* L.)"** is a record of research work done independently by Miss. Sreevidhya M.K (2007-12-101) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

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ABBREVIATIONS

AAPCD	: American Academy of Paediatrics Committee on Drugs
ADI	: Acceptable Daily Intake
AMD	: Age Related Macular Degeneration
AOAC	: Association of Official Analytical Chemists
CRD	: Complete Randomized Design
DNA	: Deoxyribo Nucleic Acid
e.g.	: Example
EFSA	: European Food Safety Authority
FD&C	: Food, Drug and Cosmetic
FD&C	: Federal Food, Drug & Cosmetic Act
FDA	: Food and Drug Administration
Fig	: Figure
FSA	: United Kingdom's Food Standards Agency
g	: Gram
GCMS	: Gas Chromatography Mass Spectrometry
GRAS	: Generally Recognized As Safe
h	: Hour (s)
HGSC	: Hyperactive Children Support Group
HPLC	: High Performance Liquid Chromatography
ITRC	: Industrial Toxicology Research Centre
JECFA	: Joint Expert Committee on Food Additives.
KFDA	: Korea Food and Drug Administration
L	: Litre
LDL	: Low-Density Lipoprotein
mg	: Milli gram
min	: Minute(s)
ml	: Millilitre
NIN	: National Institute of Nutrition
nm	: Nanometer
PCL	: Photo Chemi Luminescence
PFA	: Prevention of Food Adulteration Act
PFAR	: Prevention of Food Adulteration Rules
pH	: Hydrogen ion concentration
PMDA	: Pharmaceuticals and Medicinal Devices Agency
ppm	: Parts per million
RBC	: Red Blood Cells
Rs	: Rupees
sec	: Second (s)
SFDA	: State Food and Drug Administration
SPSS	: Statistical Program for Social Sciences
UK	: United Kingdom

US	: United States
USDA	: United States Department of Agriculture
USDHHS	: United States Department of Health and Human Services
UV	: Ultra Violet
WBC	: White Blood Cells
µg	: Micro gram
%	: Percentage
&	: And
^{0}C	: Degree Celsius

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Dedicated to
My beloved
Family

Introduction

Introduction

The most important sensory attribute of any food is its colour and it holds an important position in the overall food quality. It is one of the first characteristics perceived by the sense and is indispensable for rapid identification, preference, pleasantness and ultimate acceptance of a product (Delgado-Vargas *et al.*, 2000).

A colour additive is a dye, pigment or substance that can impart colour when added to the food, or it is a food additive which is used or intended to be used for the main purpose of colouring.

The practice of colouring food dates back to ancient times. It is known that the Egyptians coloured candy, and wine was coloured since 400 B.C. The developing food industry had availed a vast array of synthetic colours in the late 1800s. This led to colours being added for decorative purposes and unfortunately to disguise low quality foods.

Synthetic food colours are facing an uncertain future in the point of safety in use. Even permitted artificial colours if consumed indiscriminately are not completely safe. A series of legislative changes resulted in the progressive reduction in the number of synthetic colour permitted in foods. Therefore the current trend of food industry has turned to the availability of suitable natural alternatives such as carotenoids and anthocyanins, phytochemicals for many of which nutraceutical effects have been claimed, including antioxidant activity, and protect skin from damages caused by ultraviolet radiation. However the availability of natural colours, difficulties in their preparations and incorporation into food and regulating aspects are the challenges to the food industry and manufactures of natural colour. Therefore, the study of natural colourant is an extensive and active area of investigation due to the growing interest of substituting synthetic colours with toxic effect in human (Chou *et al.*, 2007).

Carotenoids are orange pigments that are naturally occurring in chloroplast of plants and in certain photosynthetic organisms like algae, fungi and bacteria. (Goodwin, 1984). There are over 600 known carotenoids, their colour ranges from pale yellow through bright orange to deep red which is directly linked to their structure. They are tetraterpenoids with 40 carbon atoms; structurally they are in the

form of a polyene chain which is sometimes terminated by rings. They are crystalline substances insoluble in water but are soluble in fat solvents, e.g., ether, acetone, etc. Carotenoids are broadly divided into two groups. Oxygenated molecules such as lutein, astaxanthin, canthaxanthin, cryptoxanthin and zeaxanthin are known as xanthophylls. The unoxygenated carotenoids such as alpha carotene, beta carotene, gamma carotene and lycopene are known as carotenes. Animals are incapable of synthesizing carotenoids and must obtain them through their diet. Carotenoids have been used as natural food colorants for centuries: saffron, pepper, annatto, and red palm oil have carotenoids as their main colour components. Besides their colouring properties, carotenoids have various health benefits such as vitamin A precursors and antioxidants leading to their wide application in the food industry (Delgado-Vargas *et al*., 2000; Klaui and Bauernfeind, 1981). Xanthophylls are one of the two classes of carotenoid pigments present in marigold, known as lutein, which are also beneficial as natural pigment source and have many commercial applications. (Varghese, 1997).

Marigold (*Tagetus erecta.* L.) is an annual herb belonging to the family *Asteraceae* is one of the important plant resources for lutein a naturally occurring yellow colouring agent, Lutein is obtained from refined marigold oleoresin a wax like dark brown solid, under heat, at 40 °C turns into viscous liquid, which is extracted from fermented marigold flowers. Its main components are oils and wax existed in plants and various carotenes mainly like lutein which can be saponified to get free lutein. It is widely used in food, pharmaceutical, cosmetic, feed and other industries as additive for its bright colour, oxidation resistance, stability, non-toxicity, lipid solubility, acidic and alkaline resistance, pigmentary properties and high safety.

This xanthophylls, like its sister compound zeaxanthin, has primarily been used as a natural colourant due to its orange-red colour found in dark green leafy vegetables such as spinach, egg yolks, various fruits, red peppers, mustard, broccoli, garden peas, leek, and kale. Lutein absorbs blue light and therefore appears yellow at low concentrations and orange-red at high concentrations. Insoluble in water, soluble in vegetable oils and solvents like ethanol, hexane and acetone. Due to lengthy synthesis lutein is not produced synthetically.

Lutein has third place in the global market of xanthophylls (Rajan, 2005). Its sales brought in more than $7 million in 2003 and saw a sales increase of 34.8 per cent. The increasing demand for marigold oleoresin, of late, could pave the way for expanding the cultivation of this flower in the country. Currently, it is used in three

applications such as poultry feed as additive; food colouring; and neutraceuticals. The potential for its increased use in the latter segment is high as studies in recent years have recommended use of lutein in food as it would help protect against two common age-related eye disorders. Since marigold is enriched with lutein, there is a big market for it. However, the major market of marigold oleoresin is Mexico where from marigold came to India and China, continues to be the major market with 50 to 55 per cent, India's share in the Rs. 300 crore world market is 25 per cent (Nair, 2005).

The two biggest trends in the food industry are health and wellness and the green initiative. The health story on the antioxidant lutein is gaining wider understanding among more consumers, according to new market research. Food fortification with lutein extract is appealing to health conscious consumers. Consumer's demand for more natural and healthier food products, presenting both nutritional and health benefits has increased over the years. So the trend for natural foods will undoubtedly become even more important in future years.

Considering the above multifaceted applications and demand for natural colours, a study was taken up with the following objectives.

1. To standardize the protocol for the separation of natural colour from marigold
2. To preserve the extract into powder/concentrate
3. Application in food and study the changes during storage

Review of literature

Review of Literature

We are in a world where colours dominate our lives, from reading signs on the road to seeing if fruit is ripe to eat. We perceive colour just as we perceive other senses like taste, smell, touch, and sound. It is the first thing we register when we are assessing anything and we make an immediate response to it before anything else. Colour is one of the most effective tools that can be used to make an impact. Colour impression can account for 60 per cent of the acceptance or rejection of any product or service.

2.1. Colour

Colour is a function of light and biology that affects our moods. It is simply light of different wavelengths and frequencies and light is just one form of energy that we can actually see that is made up from photons. Colour is the sensation experienced by an individual when energy in the form of radiation with in the visible spectrum falls up on the retina of eye. Colour is the byproduct of the spectrum of light, as it is reflected or absorbed, as received by the human eye and processed by the human brain. When light hits objects, some of the wavelengths are absorbed and some are reflected, depending on the materials in the object. The reflected wavelengths are what we perceive as the object's colour. So both as a human perception and the nature of the light absorbing chemicals that produce the light stimulus is recognised as colour (Dekker, 1999).

We are all surrounded by electromagnetic waves of energy of which colour is just a small part. The visible spectrum of colour as we see it consists of seven main colours. The seven colours of the spectrum all have varying wavelengths and frequencies. Red is at the lower end of the spectrum and has a higher wavelength but lower frequency to that of violet at the top end of the spectrum which has a lower wavelength and higher frequency

A pioneer in the field of colour, Isaac Newton in 1672 published his first, controversial paper on colour. Newton passed a beam of sunlight through a prism. The light came out of the prism was of seven different colours: red, orange, yellow, green, blue, indigo and violet. The spreading into rays was called dispersion by Newton and he called the different coloured rays the spectrum.

We see colour with the sensors in the retina of the eye called rods and cones. The rods are sensitive to low light and the cones, which require a greater intensity of light, are sensitive to colour. The message is passed to the optic nerve and then on to the brain.

2.2. Colour additive

Any material, that is a dye, pigment, or other substance made by a process of synthesis or similar artifice, or extracted, isolated, or otherwise derived, with or without intermediate or final change of identity, from a vegetable, animal, mineral, or other source and that, is capable of colouring foods, drugs, or cosmetics (FDA, 1993). A colourant appear to be coloured because they absorb some wavelengths of light preferentially. It can be both a pigment and a dye depending on the vehicle or medium it is used in.

2.2.1. Dye

By definition dyes can be said to be coloured, ionising and aromatic organic compounds which shows an affinity towards the substrate to which it is being applied. It is generally applied in a solution that is aqueous (Garfield, 2000).

Dyes are now an important ingredient in many of the medical tests (Fluorescein angiography), microbiology (Crystal violet and safranine used in Gram's stain), and cosmetics industry (hair dyes or lipsticks or nail polish shampoo). Food dyes are classed as food additives. They are manufactured to a higher standard than some industrial dyes use is strictly controlled by legislation.

2.2.2. Pigment

Pigments are generally coloured, organic or inorganic solid powder, and are usually insoluble. They are not affected physically or chemically in the substrate in which they are incorporated and can give a full range of colours. Pigments have a variety of applications that includes plastics, ink, and coating applications. They have a very special place in the paint industry**.** In the category of decorative cosmetics, the majority of colours used are pigments. It is the inorganic pigments that are popular with cosmetics but are subject to purity levels of heavy metals. The range of inorganic pigments used in cosmetics is generally made up of various chemical types (Travis, 2004).

2.3. Importance of colour in food

A food colour is any dye, pigment or substance that can impart colour when added or applied to food. Colour of a food adds to the enjoyment of eating it. The term colour additive can be applied to any dye, pigment, or other substance artificially made or obtained from a vegetable, animal, mineral, or another natural source (Branen *et al*.,

1989). We expect foods to be of certain colours, as they are in nature. We expect a peach to be orange, not blue, and lemons to be yellow, not green. Much of our acceptance of foods depends on the colours we expect.

The impression of food makes on us is a blend of sensations like, taste, flavour, colour, texture, and consistency. Colour is amongst the most important one. Characteristic colour of food is due to the pigments naturally present in it, or artificial colours added during preparation and processing to make them more attractive (Jood and Khetarpaul, 2001). Food stuff is judged by its optical view. The consumer associates freshness and quality of food with its specific strong colours. Colour provides visual information about a food's quality and condition, and influences the perception of its flavour. Everyone is sensitive to colour of food and it improves the aesthetic appeal of food. Appetite is stimulated or damped in almost direct relation to our reaction to colour. The colour we see clearly indicates the flavour we will taste. A realistic colour can indicate to the consumer a high quality product whilst a washed out or artificially bright product can indicate poor quality or an inferior product. It is said that man eats not only with his mouth but with his ‘eyes’ as well.

According to Food and Drug Administration (FDA), the primary reason of adding colours to food is to offset colour loss due to exposure to light, air, extremes of temperature, moisture and processing of food; to render characteristic flavour more recognisable; enhance colours that occurs naturally but at levels weaker than those associated with a given food; to provide a colourful appearance to certain fun foods like candies, holiday treats to create a festive appearance; to make food more appealing and appetising; to overcome natural colour variation and to ensure a consistent product; or sometimes to deceive the consumer the quality or identity of the food (FDA, 1993).

Thus the research on the objective assessment of food colours is an expanding field, since it not only to monitor the sensory quality of the product, but also to obtain rapid information about the pigments accounting for them. This is very important because evidence is accumulating that some common natural pigments such as carotenoids and anthocyanins and other flavonoids are nutritionally important because of the health benefits that it can provide.

2.4. Synthetic colours

Enhancement of colour of food stuffs goes back to Egyptian times when natural extracts and red wine were added to products such as confectionary. Accidental synthesis of the first synthetic colour, mauveine by British chemist Sir William Henry Perkin in

Germany in 1856 and subsequent commercialization made coal-tar dyes to compete with natural dyes. By the turn of the century, unmonitored colour additives had spread through the USA and Europe. Following the industrial revolution both food industry and processed food developed rapidly and unregulated colouring of food using toxic mineral and metal based compounds such as red lead, vermilion etc. Colour was also added to mask the poor quality foods, like copper arsenate in tea leaves. Advent of synthetic dyes caused rapid decline in the use of natural dyes, which were completely replaced by the former within a century (Singh, 2001).

Most colourings used today are artificial, that is, made from petrochemical coal-tar dyes. In 1900, there were no regulations on food colours. Any of over 80 dyes could be used to colour everything from cloth to candy. In 1906, the first comprehensive legislation was passed for seven colours, which were composed of known ingredients which showed no harmful effects.

The unregulated adulteration of our foodstuffs led to many deaths and serious public health concern. After the discovery of first aniline dye, synthetic dyes were freely used to a variety of food stuffs. Then doubts were raised about their safety for human consumption. The Federal Food, Drug & Cosmetic Act (FD&C) in 1938 banned many synthetic dyes for food use and made certification of the remaining ones mandatory. Safety studies showed many of the banned ones (brilliant blue, tartrazine, sunset yellow and erythrosine) to be harmful and even carcinogenic. As a result legislation came in to force to protect the consumer in particular with the Adulteration of Food and Drink Act. From late 1980s manufacturers have reduced the number and type of additives of 1960 which reduced the number of synthetic colours from several hundreds and begin to use naturally derived food colourings (Hallagan *et al*., 1995).

All colour additives permitted for use in foods are classified as certifiable or exempt from certification. Certifiable colour additives are manmade, with each batch being tested by manufacturer and FDA. This approval process, known as colour additive certification, assures the safety, quality, consistency and strength of the colour additive prior to its use in foods. Colour additives that are exempted from certification include pigments derived from natural sources such as vegetables, minerals or animals, and man made counterparts of natural derivatives (Lucas and Hallagan., 2006).

Synthetic food colours permitted in India include ponceau 4R, carmoisine, erythrosine, sunset yellow, tartrazine, indigo carmine, brilliant blue and fast green. According to Singh, (1997) Indian population consumes 220 mg of food colours per year.

Non permitted synthetic dyes commonly used in food are auramine, metanil yellow, lead chromate, sudan red, and malachite green (Bhat and Mathur, 1998).

Rao *et al*., (2005) reported that the intake of tartrazine and sunset yellow was observed to be higher during festivals due to the extensive use of these colours in sweetmeats, savouries and beverages that are most commonly available during festivals.

A study was carried out by Tripathi *et al*., (2007) to find the type and level of synthetic food colours added to various eatables in the urban and rural areas of Lucknow. Inventory of coloured eatables showed that more types and varieties of coloured eatables were prevalent in the urban areas than in the rural areas. Of the total analyzed samples, 69 percent coloured eatables revealed the presence of permitted colours while 31 percent samples contained non-permitted colours. The presence of sunset yellow and tartrazine was found to exceed the permissible limit by 8 and 20 times while in rural areas sunset yellow, tartrazine and carmoisine exceeded the permissible limit by 23, 16 and 15 times, respectively. Non permitted colours such as rhodamine B, metanil yellow, orange II, malachite green, auramine, quinoline yellow, amaranth and sudan dyes were identified in various foodstuffs. The use of these dyes is more common in the rural markets than in the urban markets.

Acceptable Daily Intake (ADI)

Synthetic food colours are permitted to be used in India under the Prevention of Food Adulteration Act, 1954 and PFA rules, 1955. Rule 30 states that any permitted synthetic colour or mixture of synthetic colours that may be added to any food enumerated in rule 29 shall not exceed 0.2g per Kg (200 ppm) of the final food. No food additive has faced more criticism and repeated screening of chemicals for toxicity than synthetic food colours.

ADI is the amount of colour that can be consumed everyday through out the life time of an individual with out any harmful effect. Joint Expert Committee on Food Additives (JECFA) examined the use of colours in foods and suggested the concept of ADI to provide the indication of safety for use. The ADI for the permitted colours varies from 0.1 mg per Kg body weight for erythrosine to 25 mg per Kg body weight for fast green (Rao *et al*., 2002). The synthetic food colours permitted in India under rule 28 of the PFA Act and their ADI are given below.

ADI of permitted synthetic colours in India (Rao *et al*., 2005)

Colour	Chemical class	ADI(mg/Kg body weight)
Carmoisine	Azo	4.0
Erythrosine	Xanthene	0.1
Ponceau4R	Azo	4.0
Sunset yellow	Azo	2.5
Tartrazine	Pyrazalone	7.5
Fast green	Triarylmethane	25.0
Brilliant blue	Triarylmethane	12.5
Indigocarmine	Indigoid	5.0

Study conducted at Industrial Toxicology Research Centre (ITRC) has revealed that 70 percent of the market sample contained non permitted colours. Food samples analysed by National Institute of Nutrition (NIN) Hyderabad showed a wide range of non permitted colours being used and permitted colours used beyond the prescribed limits (200ppm) (Tripathi *et al.,* 2007).

The recent study exploring the relationship between food colours and hyperactivity was commissioned by the United Kingdom's Food Standards Agency (FSA) by researchers at the University of Southampton in UK. This study sparked renewed attention to the possible relationship between food colour consumption and hyperactivity in children. The researchers found that two groups of children age 3 and 9 years had increased hyperactivity when they consumed two different mixtures of artificial colours plus a preservative (Mc Cann *et al*., 2007).

2.5. Ill effects of synthetic colours

Sasaki *et al*. (2002) determined the genotoxicity of 39 chemicals currently in use as food additives. Including dyes, colour fixatives and preservatives, thus more extensive consideration of food additives in current use is necessary.

Feingold, (1977) suggested that salicylates, artificial flavours, and artificial food colours were cause of hyperactivity. To prevent as well as treat hyperactivity, he recommended a diet free of these substances.

Study conducted by Singh and Singh, (2002) has showed that synthetic dyes are suspected to release harmful chemicals that are allergic, carcinogenic and detrimental to human health.

Study in Southampton general hospital in UK reported an observable effect of a combination of food colours and preservative on the behaviour of preschool children consumed fruit juices to which the artificial colours like sunset yellow, tartrazine, carmoisine, ponceau when added found allergic (Bateman *et al*., 2004).

A survey was carried out to study the quantity of consumption of coloured foods by the school children belonging to age group 13-15 years by Nayak and Nath, (2007) revealed that children are mostly attracted towards the coloured foods and are more prone to chemical insults than adults. Hence, education and awareness programmes are needed especially for children to avoid health risks due to consumption of coloured foods.

Stevenson *et al*. (2007) suggested that consumption of certain mixtures of artificial food colours and sodium benzoate preservatives are associated with increases in hyperactive behaviour in children. The study by Mc Cann *et al*. (2007) has concluded that exposure to two mixtures of four synthetic colours plus a sodium benzoate preservative in the diet resulted in increased hyperactivity in 3 year old and 9 year old children.

A case study in Hyderabad by Rao and Sudershan, (2008) about the risk assessment of synthetic food colours showed the predominant consumption of colours such as tartrazine and sunset yellow mainly from sweetmeats, beverages and fast foods while colours like carmoisine, ponceau and erythrosine were consumed by the intake of confectioneries, jams, jellies showing that the preference of colour is based on the type of foods consumed. The intakes of colours like tartrazine, erythrosine and sunset yellow were high among children due to ingestion of foods containing high concentrations of colours (9.45 mg and 4.0 mg).

Tartrazine is one of the most widely used colours in artificial foods, drugs and cosmetics. It is used in foods like ice cream, carbonated drinks. Its use is banned in Norway and Austria because of its nature to cause hyperactivity, asthma, skin rashes, and migraine headaches. Stevenson and Simon, (1981) studied about the sensitivity to tartrazine, and their result have also been implicated as they are triggering factors for asthma. Katherin and Kenneth, (1994) reported that ingestion of tartrazine cause reactions like asthma, childhood behavioural disorders, irritability, restlessness and sleep disturbance in some children.

Egger *et al.* (1985) found that mostly tartrazine and sodium benzoate generates adverse reactions like hyperactivity in children. Warrington *et al.* (1986) have reported that, in some cases of chronic urticaria food additives such as tartrazine and sodium benzoate or salicylates may play a role in the pathogenesis of the condition. These results

indicate a possible role for additive induced cell mediated immune responses in the pathogenesis of some cases of chronic urticaria.

Some scientists have studied the carcinogenetic and mutagenetic effects of tartrazine with variable results (Koutsogeorgopoulou *et al*., 1998; Walton *et al*., 1999).

The pharmacological effect of tartrazine on the gastrointestinal tract has been examined by Hutchinson *et al*. (1992) in a single *in vitro* study. Tartrazine can stimulate acetylcholine receptors in human gastrointestinal smooth muscle. This may result in an increase in fluid and chloride secretions into the bowel lumen and subsequent diarrhoea.

Walton *et al.* (1999); Sasaki *et al*. (2002) have investigated inflammatory, atrophic and carcinogenetic changes in the gastric mucosa of rats receiving tartrazine daily in their drinking water, for a prolonged period of time. A variety of immunologic responses have been attributed to tartrazine ingestion, including anxiety, migraines, clinical depression, blurred vision, itching, general weakness, heat waves, feeling of suffocation, purple skin patches, and sleep disturbance.

Amaranth found in cake mixes, wine, alcoholic drinks, fish roe etc is a water soluble azo dye used as a food colour, banned in most of the world, still used in Australia. Extreme small amounts have caused birth defects like stillbirths, foetal deaths and sterility in rats**.**

According to a report by American Academy of Paediatrics Committee on Drugs (AAPCD), amaranth cause angeoderma bronchoconstriction with ponceau and sunset yellow; erythrosine causebroncho constriction, thyroid tumor, chromosomal damage (AAPCD, 1985).

Erythrosine is an iodine containing colour used in foods, drugs and cosmetics. Found in cherries in fruit cocktail and in canned fruits for salads, confections, baked goods, dairy products, snack foods, these are banned for use in cosmetics and external drug, but not food and ingested drugs in the U.S. capable of induce cancer in man. It has been reported to be able to provoke hypersensitivity reactions (Mikkelsen *et al*., 1978; Weber *et al*., 1979; Ibero *et al.,* 1982).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1991, allocated an ADI of 0-0.1 mg/Kg body weight for erythrosine, calculated from a human study. At 3.3 mg/Kg body weight/day, erythrosine induced changes in the thyroid hormones, and above this level adenomas and carcinomas were observed in this organ (WHO, 1991). It has been proposed that erythrosine may cause acute toxicity and it has a

tumour inducer property. WHO/FDA recommends an acceptable daily intake of 100 ppm for this additive.

Combes and Havelandsmith, (1982) reviewed the genetic toxicology of the major dyestuffs used in foods, drugs and cosmetics. It has suggested that a human cancer hazard may exist when significant quantities of finished benzidine dye samples are handled. High levels of erythrosine intake can cause thyroid tumours (WHO, 1984).

Food colourants, rosebengal, erythrosine B and phloxine B are being widely used in food products such as confectionery, beverages, agricultural food products and seafood products in Japan. These colourants were found to be potential photosensitizers. So attention should be paid to these colourant containing food products at light available conditions. Especially for seafood containing high percentage of unsaturated lipids with these added food colourants, photosensitized lipid oxidation may shorten the shelf life of these food products and contribute to their quality deterioration when the light passes through transparent containers under certain conditions (Xiang-Qing *et al* ., 2005).

Indigocarmine is used in ice creams, sweets baked goods, confectionery items and biscuits. It may cause nausea, vomiting, skin rashes, breathing problems and brain tumours, DNA damage and tumours in animals. So the use is banned in the US, Japan, Australia and Norway. UK use restricted to maximum permitted levels. Severe bradycardia can occur after intra-arterial administration of indigo carmine during general anaesthesia (Satoh *et al*, 2001).

Graziano *et al*. (2005) reported a case of severe hypotension and bradycardia following intravenous indigo carmine injection in a patient with known sulfa allergy. Intravenous administration of indigo carmine was immediately followed by the development of second degree anti ventricular block. There are evidences to indicate that exposure to indigo carmine during pregnancy may cause birth defects. The likelihood and severity of defects may be affected by the level of exposure and the stage of pregnancy that the exposure occurred. Indigo carmine is harmful to the respiratory tract if swallowed. It is also an irritant to the skin and eyes.

Ponceau 4R is a red synthetic coal tar or azo dye found in dessert toppings, jelly, salami, seafood dressings, tinned strawberries and fruit pie fillings and packet cake mixes, cheesecakes, soups, carbonated drinks ice creams, confectionery items and desserts. It is banned in US, Canada, Norway, Sweden and Japan. Use is restricted to maximum permitted levels in the UK.

It appears to cause allergic and intolerance reactions particularly amongst those with aspirin intolerance or asthmatics. It can produce cancer, DNA damage and tumours in animals. Not recommended for consumption by children. The Hyperactive Children Support Group (HGSC) believe that, a link exists between these additive and hyperactive behavioural disorders in children (McCann *et al*., 2007).

Metanil yellow widely used in food items like ladoos cause insufficient oxygen supply to skin and mucus membrane along with degenerative changes in stomach, liver, kidney, abdomen and testes. It is the principal non permitted food colour used extensively in India. The effects of long term consumption of metanil yellow on the developing and adult brain in rats (Nagaraja and Desiraju, 1993). In the treated rats the amine levels in the hypothalamus, striatum and brain stem were significantly affected. These effects on major neurotransmitter systems indicate that chronic consumption of metanil yellow can predispose both the developing and the adult central nervous system of the rat to neurotoxicity.

Metanil yellow, a non permitted food colour, has been found in various foodstuffs. Oral administration of metanil yellow 430 mg/Kg body weight to four animals for seven days caused significant induction of hepatic *P*-450 and its dependent aryl hydrocarbon hydroxylase activity and cytosolic activities (Das *et al.*, 1997).

Lead chromate powder investigated as a tumorigen and mutagen causes human reproductive hazards. It is added as a colourant to chilli powder results in epigastric pain, anaemia, nausea and constipation due to lead toxicity among Ghurkha soldiers (Power *et al.*, 1969).

Rhodamine B exerted fewer toxic effects in rats. The growth of rats fed one percent Rhodamine B for 90 days was inhibited to a greater extent than that of rats fed 3,6-diaminofluoran at the same dietary level over the same period of time. Liver enlargement, the most significant abnormality at autopsy, occurred to a greater extent in the rats fed Rhodamine B (Webb *et al.,* 1961).

Sunset Yellow, Orange yellows which is banned in Norway, Sweden and Finland. Restricted to maximum permitted levels in U.K. Their presence in sweets snack foods ice creams, yoghurts, drinks etc causes growth retardation and severe weight loss in animals, allergies and asthma, cancer, DNA damage, nasal congestion, hyperactivity, kidney tumours, abdominal pain, nausea and vomiting, indigestion, distaste for food, increased incidence of tumours in animals.

Helal *et al.* (2003) studied the interaction between food preservative sodium nitrite (NaNO3) and food colourant sunset yellow. Ingestion of NaNO3 and sunset yellow mixture significantly decreased rat's body weight, RBC and WBC counts, serum inorganic phosphorus, serum protein and serum albumin in rats. Significant increases were observed in serum glucose, calcium, pH and cholesterol. Also cholesterol of brain, liver and heart were significantly elevated. A complete recovery of most biochemical and haematological parameters was observed days after stoppage of the mixture. The results showed that even the permitted colourants and food preservatives when taken together or if taken in excessive quantity may be harmful.

The Food Advisory Consumer Service in South Africa warns about the use of sudan red1, also known as yellow 14. It is an industrial, red, soluble dye used legally to colour a number of non food products such as waxes, floor polishes and solvents. It has been shown to cause cancer in rats and, although its use is not allowed in foods, the dye has been found in food products such as chilli powder. Related dyes that are similarly misused are sudan 2, 3 and 4. Because of structural similarities to sudan I, would be careful to assume that they are potentially genotoxic and possibly carcinogenic (Brantom, 2005).

Spices Board of India formulated a mandatory testing programme in all chilli consignments exported from India. Hence chilli powders were monitored to check the magnitude of artificial colouration and the likely exposure assessment of sudan dyes. Among 800 non branded, loose chilli powder samples, over 66 percent were found to employ artificial colouration. The maximum content of sudan I noted was as high as 11.8 mg/g, which at the rough per capita consumption estimates of 0.5-1.0 g chilli powder per day amounts to an intake of 5.8-11.8 mg of sudan I. This may lead to unwarranted health consequences.

Commission Working Group on the Classification and Labelling of Dangerous Substances considered sudan I in 2001 and on the basis sudan dyes produce kidney lesions. Publications classified this dye as a dermal sensitizer (R43) is advisable to safeguard the health of unsuspecting consumers (Mishra *et al*., 2007).

Malachite green consisting of green crystals with a metallic lustre is highly soluble in water and is highly cytotoxic to mammalian cells and also acts as a liver tumour promoter (Fernandes *et al*. 1991; Rao and Fernandes, 1996). It is toxicologically classified under category III and has been identified as harmful by the WHO/FAO Committee. It is used extensively for dyeing wool, jute, cotton, and leather, as a

laboratory reagent and as a topical antiseptic. It occurs as a non permitted food colouring agent. Study by Mahudawala *et al*. (2000) indicated its relationship between increased cell proliferation associated with the malignant transformation of Syrian hamster embryo.

Red is obtained from mercury sulphite, which causes skin cancer, paralysis, impaired vision, blue from Prussian blue, silver from aluminum bromide both being carcinogenic, green from copper sulphate, which cause eye allergy, puffiness and temporary blindness, black from lead oxide causing renal failure and learning disability.

Prasad and Rastogi, (1983) found that feeding of albino mice in the common food colour material yellow led to changes in haematological values. The short term toxicity of this dye is characterized by the induction of met haemoglobin anemia and increased red blood cell turnover. Activation of genotoxic metabolite may occur in mammalian systems.

2.6. Need for natural colours

Natural food colour is any dye, pigment or any other substance obtained from vegetable, animal, mineral, or source capable of colouring food drug, cosmetic or any part of human body, colours come from variety of sources such as seeds, fruits, vegetables, algae and insect. Natural pigments offer an alternative to synthetic dyes. The continuing growing public demand is for natural and less chemical. Complimenting their colouring effect, many natural colours can provide nutritional and health benefits**.** The involvement of synthetic dyes in carcinogenesis makes natural dyes, which are harmless and, what is more, useful, preferable for food and pharmaceutical industries (Sarafanova and Dobavki, 2003)

From the last twenty years, food sector is experiencing a trend back towards natural colourants (Downham and Collins, 1999). The consumers are more concerned over possible health risks associated with synthetic food additives and there is strong demand for more natural products.

Synthetic colourants possess an intensive colouring strength, good solubility, high stability and they are easier to process than natural food colourants. The application of natural food colourants is limited and they have some disadvantages compared with the artificial colourants, though they are relatively safe in terms of toxicological check and incompatibility reactions (Abd El-Galeel, 2002).

Acceptability

Colour is an important factor in the acceptability of food products and food quality is first adjudged on the basis of its colour. People associate certain colours with

certain flavours (Delwiche, 2004) and the colour of food can influence it perceived flavour. In fact, the colour of a food influences not only the perception of flavour, but also attraction and quality and subsequently, consumption.

Food manufacturers therefore often add colourings to their products to simulate or enhance a colour that is perceived by the consumer as natural, to mask natural variations in food colours, to offset colour loss due to light, extremes of temperature, moisture and storage conditions. In addition food colourants provide identity to foods, by colour impartation on foods which would otherwise be colourless. And sometimes they are added just for effect or decorations purposes (Henry, 1996; Food Advisory Committee, 1987).

Safety in use

Recent controversy and criticism about the safety of food colourants have been directed towards the synthetic colourants. The natural colourants have been relatively free of criticism may be due to the belief that most are derived from food sources that have been consumed for many years (Francis, 1989). Natural dyes are less toxic, less polluting, less health hazardous, non-carcinogenic and non poisonous. Nowadays there seems to be an increasing awareness among people towards natural products. Due to their non-toxic properties, low pollution and less side effects, natural dyes are used in day-to-day food products.

Health benefits

Beside the colour attributes, anthocyanins and other phenolic colourants from plant sources have been reported to be beneficial to health with potential physiological effects, such as antineoplastic, radiation protective, vasotonic, vaso-protective, anti-inflammatory, chemo and hepato protective effects (Kamei *et al*., 1995; Mazza and Miniati, 1993; Minkova *et al*., 1990; Wang *et al*., 1997).

Natural dyes are environment friendly. Turmeric, the brightest of naturally occurring yellow dyes is a powerful antiseptic which revitalizes the skin, while indigo gives a cooling sensation. (Mahanta and Tiwari, 2005). Due to perceived safety and physiological advantage of the natural colourant over synthetic ones, interests are being geared into search of new natural colourants and the verification of the safety of existing ones.

2.7. Natural colours

Natural colours are colours that have been derived from agricultural or biological materials using conventional methods and do not require certification (Williamson, 1995). They are usually exempt from certification by FDA. Annatto extract, dehydrated beets, caramel, carmine, grape skin extract, fruit and vegetable juices, paprika, saffron, xanthophylls and turmeric. They are widely used in variety of food including meats, cheeses, ice creams, yogurt, syrups, baked goods and beverages. Above all, they are environment friendly and can be recycled after use.

Tedious extraction of colouring component from the raw material, low colour value and longer time make the cost of dyeing with natural dyes considerably higher than with synthetic dyes. Thus the study of natural colourants is an extensive and active area of investigation due to the growing interest of substituting synthetic colourants with toxic effects in human (Chou, 2007).

Indians are considered as forebears in the art of natural dyeing. Natural colours find use in the colouring textiles, drugs, cosmetics, etc. Due to their nontoxic effects, they are also used for colouring various food products. In addition to their dye yielding characteristics, some of these plants also possess medicinal value. Though there is a large plant resource base, little has been exploited so far. Due to lack of availability of precise technical knowledge on the extracting and dyeing technique, it has not commercially succeeded like the synthetic dyes (Siva, 2007).

Chandramouli, (1995) reported that plants are dominated sources of natural dyes, producing different colours like red, yellow, blue, black, brown and a combination of these. Almost all parts of the plants like root, bark, leaf, fruit, wood, seed, flower, etc. produce dyes. Over 2000 pigments are synthesized by various parts of plants, of which only about 150 have been commercially exploited. Nearly 450 taxa are known to yield dyes in India.

Singh *et al*. (2005) studied the antimicrobial activity of some natural dyes. Optimized natural dye powders of *Acacia catechu L., Kerria lacca, Rubia cordifolia L.* and *Rumex maritimus* were obtained from commercial industries and they showed antimicrobial activities. This is clear evidence that some natural dyes by themselves have medicinal properties**.**

Some important natural colours and their source are given below (Siva, 2007).

Name	Colour	Source
Carmine	Red	Cochineal insect
Betanin	Red	Red beetroot
Capsanthin	Orange/red	Paprika
Anthocyanins	Red/purple	Red cabbage,black grapes, hibiscus
Annatto	Red/ purple	Annatto seed
Curcumine	Yellow	Turmeric
Crocetin	Yellow	Turmeric
Lutein	Orange	Marigold
Lycopene	Orange/red	Tomato
Beta carotene	Orange	Carrot oil
Chlorophyll	Green	Green leaf plants
Caramel	Brown	Caramelised sugar
Carbon	Black	Black carbon vegetable material

Chlorophyll

Green colour found in plants and the most widely distributed natural plant colour. Because of their colour and their physico chemical properties, they are also used as additives to food products (Schoefs*.,* 2002). Natural chlorophyll is insoluble in water but soluble in organic solvents like acetone, alcohol and ethyl ether. Treatment with alkali produces the water soluble chlorophyllin. They are not very stable to light and heat. Chlorophyllin is unstable to acidic conditions. Chlorophyll has antioxidant and chlorophyllin has anticancerous and antibacterial action. It is used in food industry (yogurt and ice creams), pharmaceuticals, soaps and detergents and also in cosmetic industry.

Curcumine

Golden yellow pigment extracted from the tuber of the plant turmeric (*Curcuma longa*). The dried tubers are ground and the oleoresin is extracted by solvent extraction. Oleoresin contains 55-80 percent curcumine. Ethylene dichloride is an excellent solvent for curcumine. Other solvents are methylene dichloride and ethyl acetate (Varghese, 1999). Pure curcumine is insoluble in water so it is dissolved in food grade solvents and permitted emulsifier.

Turmeric is a golden coloured spice commonly used in the Indian subcontinent, not only for health care but also for the preservation of food and as a yellow dye for textiles. Curcumine gives the yellow colour to turmeric, was first isolated almost two centuries ago. Since the time of Ayurveda (1900 BC) numerous therapeutic activities have been assigned to turmeric for a wide variety of diseases and conditions, including

those of the skin, pulmonary, and gastrointestinal systems, aches, pains, wounds, sprains, and liver disorders (Aggarwal *et al*., 2007).

Carmine

Carmine brilliant red dye produced from insects living on cactus plants. When mixed with water, the powder produced a deep, vibrant red colour. Most cherries today have a bright red appearance through the artificial colour 'carmine'. The most stable of all naturally derived colours, with excellent stability to both heat and light. Derived from the dried cochineal insect (*Dactylopius coccus*) the colouring substance is carmic acid which has a pale yellow colour but complexes readily with tin, aluminium, and other metals in to brilliant red pigments. Approximately 70,000 insects are required to produce one pound of dye. The light and heat stability of the carmine makes it ideal for food products exposed to direct sun light.

Betalaines

These are red water soluble pigments found in beet root, cactus fruit, and poke berries. Pocess antioxidant and radical scavenging properties (Kanner *et al*., 2001). The extraction method used is pressing using hydraulic press. The extract is then filtered and vacuum concentrated and spray dried. It is applied in ice creams, yogurt, soft drinks and bakery products

2.9.1. Carotenoids

Carotenoids form a group more than 600 fat soluble compounds that are biosynthesised mainly by higher plants but also by some yeast, fungi, algae and bacteria by condensation of isoprene units (Goodwin, 1984). Carotenoids are responsible for a variety of bright colours in fall leaves flowers (narcissus, marigold), fruits (pineapple, citrusfruits, paprika), vegetables (tomato, carrots), insects (ladybird), bird plumage and animals (crustaceans, salmon) (Pfander, 1992). Rodriguez-Amaya, 1997 reported that, carotenoids are the most abundant micronutrients in vegetables and fruits. Beta carotene is a popular choice for use in baked goods exhibiting excellent colour retention properties and normal fading (Gordan, 1997).On world wide basis about 60 percent of vitamin A is estimated to come from pro vitamin A carotenoid in developing countries they contribute up to 82 percent. Carotenoids furnish flowers and fruits with distinct colours ranging from yellow to red and are essential components for photosynthesis (Niyogi, 2000).

Some important carotenoids and their dietary sources

Alpha, beta and gamma carotene	:	Carrot, papaya pumpkin, mango, spinach
Lycopene	:	Tomato, red chillies
Lutein	:	Spinach, bell pepper, orange, marigold, egg
Zeaxanthin	:	Orange, bell pepper, sweet corn, egg

The leafy vegetables are a very good source of lutein and beta while the fruits have a considerably lower content of carotenoids (lutein, 0.0032–0.16 mg/100 g and beta carotene, 0.010–0.17 mg/100 g) and a complex and variable qualitative and quantitative carotenoid composition (Dias *et al*., 2009).

Carotenoids are classified into two types of compounds, carotenes and xanthophylls. Carotenes are unsaturated hydrocarbons, e.g. lycopene and beta carotenes. Xanthophylls have one or more functional groups containing oxygen, e.g. lutein, astaxanthin, and canthaxanthin. The former present a hydrophobic character, whereas the latter can provide a certain polar character (Borel *et al*., 1996). Carotenes and xanthophylls are closely related in molecular structure, and the term 'carotenoids' is commonly used to refer to the entire group of diverse, and yet closely related substances (Krinsky *et al*., 2003)

The conjugated double bond system constitutes the light absorbing chromophore that gives carotenoids their attractive colour and provides the visible absorption spectrum that serves as a basis for their identification and quantification. The ability of carotenoids to absorb light arises from the presence of a conjugated polyene chain. The wavelength maximum of the absorption band is related to the extent of the conjugation in the polyene chain (Kohler, 1995).

They are insoluble in water and soluble in organic solvents, such as acetone, alcohol, ethyl ether, chloroform, and ethyl acetate. Readily soluble in petroleum ether, hexane, toluene, methanol and ethanol. Crystalline carotenoids may be difficult to dissolve in the above solvents but do dissolve in benzene and dichloromethane (Schiedt and Liaaen-Jensen, 1995). Solubility of both beta carotene and the xanthophylls lutein in tetrahydrofuran was shown to be excellent (Craft and Soares, 1992)

Carotenoids are pigments found primarily in plants. Carotenoids, long recognized for their antioxidant properties, are of increasing interest in relation to cancer because of their effect on regulation of cell growth, modulation of gene expression, and, possibly, immune response (Rock, 1997).

Gordan, (1997) have reported the current aspects of food colourant from carotenoids and its broad commercial use, which has generated further interest in marigold as an alternative crop. Carotenoids and anthocyanins are amongst the most utilised vegetable carotenoid in the food industry. Carotenoids also play an important role in human nutrition and health, participating in pro vitamin A and anti cancer activities (Fraser and Bramley, 2004).

Plant constituents such as carotenoids and flavonoids are involved in protection against excess light in plants and contribute to the prevention of UV damage in humans. Carotenoids are suitable compounds for photoprotection in the human. In addition to beta carotene, other carotenoids like lycopene or lutein can be used as photoprotectants (Stahl and Sies, 2007).

Annatto

Red colour obtained from the seeds of annatto **(***Bixa orellana L.*) used in cheese, fish processing, confectionary, and other dairy products (Satyanarayana *et al*., 2006). The colour of annatto is due to several apocarotenoids, in which bixin is the most important. Fruits are dried, threshed to remove the seeds from pods. It is extracted to get oil soluble or water soluble forms, bixin and nor bixin. It will fade up on exposure to strong, direct sunlight, susceptible to degradation by oxidation, but are resistant to microbial attack.

Paprika

It is the deep red coloured non pungent dried fruit of *Capsicum annum* (Anu and Peter, 2000). The colour in paprika is due to carotenoids namely capsanthin and capsorubin. It is a natural colour source excempt from certification in USA and they can be used directly (Marmion., 1979). The outer pericarp of paprika is the main source of capsanthin and capsorubin which impart appealing colour aesthetic flavour and aroma has many end use in various food, pharmaceuticals and cosmetics. Paprika oleoresin is insoluble in water while readily soluble in vegetable oil. One main reason for high demand for paprika pigment is to incorporate in poultry feed, results in reddish tint to the chicken meat and red egg yolk which is more appealing to the consumers. Prasanth and Ponnuswami, (2008) developed high colour chilli varieties for extractable colour and through breeding studies.

Saffron

Saffron is a luxurious and expensive natural ingredient used as food colour and flavour. Very intensively fragrant, slightly bitter in taste. By soaking it in warm water gets a bright yellow- orange solution, which is caused by the carotenoids, especially crocetin

esters. The plant part used is the stigma of flowers, about 170,000 flowers are needed to make 1Kg dried saffron. It is extracted to make saffron extract powder. Tsimidou and Tsatsaroni, (1993) successfully extracted a crocin from saffron only cold water, since the pigment is water soluble.

2.9.1.1. Anthocyanins

Anthocyanins provide natural colours for food industry in red, violet and blue spectrum. There are numerous compounds belonging to this group, only a handful possess the specific chemical structures which maximize their stability and hence their suitability for extraction. It can be easily incorporated in aqueous food system because anthocyanins are highly soluble in water and alcoholic solutions. When in water they are more stable at low pH (Pascual-Teresa and Sanchez-Ballesta, 2008). Red grapes elderberries, red cabbage, blood orange, black chokeberry and sweet potato are some sources of anthocyanins (Bridle and Timberlake, 1997). They are extracted by infusing the chopped or crushed fruit or vegetable with water acidified with common food acid then concentrated by non chemical separation techniques.

Lycopene

It is a bright red carotenoid pigment found in tomatoes watermelon, carrot and other red fruits. It is insoluble in water, used in food, beverages, cosmetics, pharmaceuticals and other applications. It is also used as a colour ingredient in many food formulations. It has received considerable attention in recent years because of its possible role in the prevention of chronic diseases such as prostate cancer (Clinton, 1998; Rao and Agarwal, 1999). Epidemiological studies of Giovannucci, (1999) have also shown that increased consumption of lycopene-rich food such as tomatoes is associated with a low risk of cancer. Lycopene is the precursor to bixin and norbixin, pigments from *Bixa orellena*, commonly used for colouring foodstuff. Lycopene has antioxidant properties that may reduce the incidence of certain cancers (Shi *et al.*, 1999). Lycopene is the most important pigment in tomato. Which are usually extracted with organic solvents such as chloroform, hexane, acetone, petroleumether. Supercritical fluid extraction has been proposed to obtain a high purity lycopene extract. The obtained carotenoid rich extract is usually used in health foods, food additives, medicines and cosmetics.

2.9.2. Xanthophylls

Xanthophylls are one of the two classes of carotenoid pigments. It is present in marigold, known as lutein, which are also beneficial as natural pigment source and have many commercial applications (Varghese, 1997). Zeaxanthin and antheraxanthin, other

members in the family, are also found to be involved in heat (energy) dissipation by converting themselves to violaxanthin, thus adding additional measures for the protection of photosynthesis systems. The richness of xanthophylls in marigold made it to be recommended as raw material for the lutein pigment by FAO.

2.9.2.1. Marigold

Marigold (*Tagetes erecta*. L) belongs to family Asteraceae. The genus *Tagetes* is recognised as source of natural colours (Timberlake and Henry, 1986). It is the richest source of xanthophylls mainly lutein found in nature. It is native plant from Mexico and has been used in traditional Mexican medicine (Neher, 1968). Dried and ground marigold flowers have been used commercially since 1966 as botanical ingredients in animal feeds. And since 1969 as starting materials for the production of marigold extracts, which contain xanthophylls esters as the commercially important component (Ausich and Sanders, 1997; Levi, 2001).

The common term 'marigold' means a diversity of plants with golden flowers, including the *Tagetes* species of the Americas. It has been widely introduced throughout the world, some purely for decorative purposes and others for industrial use. *T. glandulifera* Schrank and *T. minuta* L. are cultivated in southern Africa and India in addition to South America for the production of tagetes oil, an essential oil employed by the international perfumery industry. World demand for this oil is about 10 tonnes annually. Another species, *T. erecta* L. or 'Aztec marigold' provides an important yellow colourant from its flowers. The principal pigment in the flowers is the xanthophyll, lutein, which is present in the form of esters of palmitic and myristic acids (Anon, 2004).

Early Anglo-Saxons called the Marigold 'Golds' or 'Ruddes' and flowers were often boiled to extract their yellow colour for food colouring, fabric, and even hair dyes. After extraction, a yellow powder is produced (Paquette, 2007).

Kasemsap *et al.* **(**1990) analyzed 22 marigold cultivars for xanthophylls and carotene content, among them *Tagetus erecta* was identified as one of the cultivars suitable for obtaining dye. *Tagetes patula* and *T. erecta* petals are rich sources of carotenoid esters, which contain lutein esters (Breithaupt *et al*., 2002; Hadden *et al*., 1999).

Petals of marigold are rich in lutein and lutein fatty acid esters which on the whole represent over 90 percent of the pigments identified in the plants (Quackenbush, 1973). According to the study by Piccaglia *et al*. (1998), flowers with orange and deep orange petals were found to have higher concentrations of pigments than those with

yellow or green yellow petals, and 97 percent of the determined pigments were in petals. Relevant quantitative differences were found among the marigold types which had a total content of pigments ranging from 17 to 570 mg/100 g in the petals and from 0.4 to 18.6 mg/100 g in the calyces. Shewmaker, (2002) reported that only marigold flower petals, which have higher xanthophylls content than the remaining flower parts.

The results of preliminary investigation performed by Deineka *et al.* (2005) showed that the maximum amount of xanthophylls was accumulated in the flowers with orange petals, where the content of carotenoids exceeded 5 mg per gram of fresh petals, against 1 mg/g for yellow and 0.2 mg/g for lemon-yellow flowers.

Marigold yellow colour is a carotenoid rich oil soluble colour obtained from the petal. Its principal ingredient is lutein esters. Among carotenoid rich food colours, lutein ester has a brighter yellow colour than Annatto food colour and beta carotene. Marigold flower which are yellow to orange red in colour, are rich source of lutein, a carotenoid pigment. Dimyristate and dipalmitate were reported as the major diesters of lutein (83–92 percent of total esters) in marigold flower (Philip and Berry, 1975). With the application of new innovations, natural pigments can become more cost effective and increase their competitiveness against certified dye and dye products (Soubhagya *et al.*, 2004).

Although many fruits and vegetables contain lutein, the best commercial source of pure lutein is marigold (*Tagetes erecta L.*). The majority of lutein esters in marigold are diesters due to the two hydroxyl groups, one at each ionone ring**.**

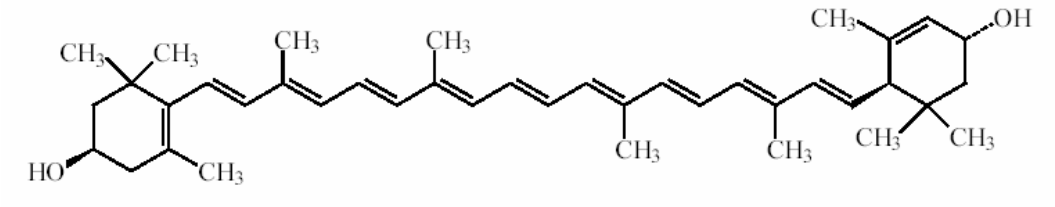
2.9.2.2. Lutein

Lutein from *Tagetes erecta* L. is a purified extract of xanthophylls obtained from marigold oleoresin. The oleoresin is prepared from hexane extracts of marigold flowers, saponified with potassium hydroxide in either methanol or propylene glycol. The resulting crystalline material contains lutein, and minor components including other carotenoids and waxes. (Cantrill, 2004)

Extracts of marigold flowers, fresh raw kale, corn meal, spinach, and human plasma also contain small amounts of cis isomers of lutein (Krinsky *et al*., 1990; Khachik *et al*., 1999). Lutein is commonly isolated from marigold oleoresin together with zeaxanthin

Chemical and physical properties of lutein.

The principal colouring component of marigold flower is lutein, a fat soluble carotenoid (C40H5602 (3, R, 3'R, 6R)-/3, &-3, 3'-diol) (Quackenbush and Miller 1970; Gau *et al.,* 1983; Rivas 1989).



Chemical structure of lutein and zeaxanthin lutein (C40H56O2). Formula weight 568.88.
(Krinsky *et al.,* 2003)

Lutein and zeaxanthin are both isomeric dihydroxy-carotenoids with the ionone ring systems being substituted at both the 3 and 3' carbon. The presence of the hydroxyl groups makes lutein and zeaxanthin distinctly more polar than their respective carotene analogue and beta carotene (Craft, 1992). The presence of the hydroxyl groups at both the 3 and 3' carbons suggests that a close similarity in physical properties exists between lutein and zeaxanthin. Lutein is soluble in non polar or dipolar solvents such as hexane, benzene, ethers, methylene chloride, chloroform and alcohols.

The ability of carotenoids to absorb light is due the presence of a conjugated polyene chain. The maximum wavelength of the absorption is related to the extent of the conjugation in the polyene chain (Kohler, 1995). Luteins have nine conjugated double bonds in the polyene chain. Lutein has an absorption maximum of 445 nm in ethanol.

Since the food colour is extracted from the petal and refined, it lacks a peculiar smell and can be used easily in food materials. Compared with beta carotene and annatto, this colour has a strong yellow colour. This colour easily dissolves in oil and fat, compared with beta carotene. It has outstanding acid resistance and can be used in acidic food. Heat resistance and light resistance are also relatively stable, and the effect of pH is negligible.

Food sources of lutein (Huck *et al.,* 2000)

Food	Lutein content (µg/100g wet weight)
Broccoli	2358
Kale	6390
Carrot	280
Spinach	3920
Tomato granulate	226
Tomato powder	39
Tomato flakes	99

Although lutein is one of the major constituents of many green and yellow-orange vegetables and fruits, the isolation and purification of pure lutein in large quantities from

these foods is not economical, as many time consuming purification steps are required to remove and purify lutein from other components, including other carotenoids present in these foods.

2.9.2.3. Importance

Lutein is an antioxidant which is believed to be an essential nutrient for normal vision. The protective role of lutein against eye damage is well documented. Studies have also indicated that lutein improves heart health, protects our skin against UV damage, reduces diabetes induced oxidative stress, and possesses anti inflammatory and anti cancer properties.

2.9.2.3.1. As food colourant

Lutein, extracted from *Tagetes erecta* and from other species of edible plants, is already permitted for use as a food additive by European Food Safety Authority, (EFSA)**.** Lutein has previously been evaluated as a food colour by the Scientific Committee on Food (SCF), which concluded that as long as it was derived from food sources and intake would not exceed what could be expected from the consumption of normal food, the use of the colour in food would be acceptable (SCF, 1974). Lutein is accordingly permitted in foods in the European Union (EU), and the specifications of lutein may, beyond some food sources also be extracted from *Tagetes erecta*. According to new specifications prepared at the 63rd Joint FAO/WHO Expert Committee on Food Additives (JECFA), ADI of 0-2 mg/Kg body weight for lutein from *T. erecta* and synthetic zeaxanthin was established (JECFA, 2004).

Food colourants are tested for bio safety before its promotion and are controlled by various regulatory bodies around the world and regulation varies in different countries (Hallagan *et al*., 1995). In US, FD&C (Food, Drug and Cosmetic) numbers are given to synthetic food colourants approved by FDA that do not exist in nature, while in EU, E numbers are used for all additives of food applications. Thus, the approved list of food colours varies along the countries, it means each country has its own approved list, including limit of maximum daily intake. Out of them, some other regulatory agencies are there like PMDA (Pharmaceuticals and Medicinal Devices Agency) in Japan, SFDA (State Food and Drug Administration) in China, CDSCO (The organization and function of the Medicines Agency) in India and KFDA (Korea Food and Drug Administration) in South Korea etc. Most of the food grade bio colourants approved by FDA or EU are also approved by other agencies. For example, in India, Rule 26 of The Prevention of Food Adulteration Rules (PFAR) permits 11 colours for food use (Chattopadhyay *et al*., 2008).

Lutein is used as a colourant in pea protein stabilized food emulsions (Raymundo *et al.*, 2002). Lutein was also incorporated in salad dressing as a dietary compound that may help delay the onset of age related macular degeneration (AMD) associated with legal blindness (Losso *et al.*, 2005). Jones *et al*. (2005) incorporate lutein in to Cheddar cheese. It was used as colourants in reduced fat mayonnaise containing spent brewer's yeast (Santipanichwong and Suphantharika., 2007).

2.9.2.3.2. Eye protection

The central part of the retina, called the macula, contains macular pigments in which lutein is concentrated. The yellow coloured pigments protect the retina from damage of the photo oxidative affect of high energy light. Lutein offers eye protection by lowering the risk of age related vision loss, which causes gradual loss of central vision. Age related vision loss or age related macular degeneration (AMD) is caused by steady damage of the retina especially affecting elderly people (Stone *et al*., 2001; La Cour *et al*., 2002).

Wald, (1945) reported that yellow spot in the macula of human retinas might be a carotenoid, appeared. He dissected the foveal region of 10 human retinas, extracted them with chloroform, and reported that the spectrum of the yellow pigment agreed quite well with the visual estimate of the macular pigment, derived from the differences in the log sensitivity of peripheral and foveal cones. Fifty years after this observation, carotenoids were also identified in the lens of the human eye (Yeum *et al*., 1995) and several years later, carotenoids were identified in virtually all of the tissues of the eye (Bernstein *et al*., 2001). These carotenoids are concentrated in the inner retinal layer of macula, where the concentration is high and variable (Bernstein, 2002).

Chromatographic characterization of the macular pigment was done using a high performance liquid chromatography (HPLC) analysis to demonstrate that there were actually two xanthophylls present in macula, namely lutein and zeaxanthin. Higher levels of lutein and zeaxanthin were measured in the retina of individuals without known clinical history of AMD while lower levels of both xanthophylls were measured in the eyes of individuals with known clinical history of AMD (Bone *et al*., 2001).

Lutein and zeaxanthin have been identified and recognized by various interdisciplinary studies as one of the dietary strategies that can delay the onset of macular degeneration (Seddon *et al*., 1994; Beatty *et al*., 1999; Berendschot *et al*., 2000). It has been suggested that eating leafy vegetables, which are rich in lutein and zeaxanthin, may decrease the risk for AMD (Curran-Celentano *et al*., 2001). They are the only

dietary carotenoids present in the macular region of the retina and the lens (Bone *et al*., 2001). It was suggested that 6 mg of lutein a day may reduce the risk of AMD by 43 percent (Seddon *et al*., 1994). Findings of Bhosale *et al*., (2009) support current recommendations to increase dietary intake of xanthophyll carotenoids in individuals at risk for AMD.

2.9.2.3.3. Antioxidant

Lutein is an antioxidant that appears to reduce harmful free radicals in various parts of the body. Free radicals can play a role in a variety of chronic diseases. It filters the high energy, blue wavelengths of light from the visible light spectrum by as much as 90 percent. Blue light, in both indoor lighting and sunlight, is believed to induce oxidative stress and possible free radical damage in human organs exposed to light, such as eyes and skin. Inhibit auto oxidation of cellular lipids Zhang *et al.* (1991) and also protect against oxidant induced cell damage (Martin *et al*., 1996).

There are 8 carotenoid metabolites and one stereoisomer in human serum or plasma that result from a series of oxidation reduction reactions of three dietary carotenoids: lutein, zeaxanthin and lycopene. These metabolites were first isolated and characterized by (Khachik *et al*., 1992)

Xanthophylls have shown to be effective as free radical scavengers (Krinskey, 1994). Epidemiological studies have indicated an association between high vegetable intake and a lower risk of chronic degenerative diseases such as certain types of cancer, cardiovascular diseases.

There has been recent evidence to suggest that lutein, one of the most abundant carotenoids in the diet and in human blood, possesses strong antioxidant capabilities and therefore may be useful in reduction of the incidence of cancer Chew *et al*. (1996); Hadden *et al*. (1999) reported the potential benefits of lutein which include cancer prevention and enhanced immune function. It suppresses mammary tumour growth and enhances lymphocyte proliferation.

The antioxidant activity of lutein was examined by Wang *et al*. (2006) using the photochemiluminescence (PCL) assay. Lutein showed a greater antioxidant activity than the other two common carotenoids, beta carotene and lycopene. The mutagenicity and anti-mutagenicity of lutein were examined. Lutein was not only found to be non mutagenic at all doses, but it showed an anti mutagenic effect in a dose dependent manner. Similar results were found in a chromosome aberration test using Chinese hamster ovary cells for the evaluation of clastogenicity and anti-clastogenicity of lutein

their findings provided scientific evidence for the safe use and health beneficial effects of lutein.

Lutein can also reduce the risk for artery diseases. Studies have shown that persons with the highest lutein intake showed the lowest artery wall thickening. Lutein reduces the oxidation of LDL cholesterol thereby reducing the risk of artery clogging. Prevent cardiovascular sclerosis, coronary heart diseases resulted in aging body. Protects arteries and the lungs from damaging free radicals inhibit arthritis, skin cancer and tumour. It can be widely used in cosmetic products, fodder, medicine, aquatic products and other industries

2.9.2.3.4. As feed additives

Most consumers associate colour of food with age and health status of animals and food quality in consequence. Xanthophylls are widely used as feed additives to generate products meeting consumer's demands. Although a lot of xanthophylls with interesting biological properties are found in nature, only a few are actually of industrial importance as feed additives.

Lutein was traditionally used in chicken feed to provide the yellow colour of broiler chicken skin (Bletner *et al*., 1966; Tyczkowski and Hamilton, 1991; Hencken, 1992; Bernhard *et al*., 1997; Picaglia *et al*., 1998; Levi, 2001., Subagio and Morita, 2001). Feed colourants because of their many advantages, such as safety, nontoxicity, strong biological activity, and greater bioavailability (Liu *et al*., 2008). Such lutein fortification also results in a darker yellow egg yolk. Today the colouring of the egg yolk has become the primary reason for feed fortification.

Alam *et al*. (2006) determined the deposition of the pigments in the egg and tissue of laying hens fed marigold petal. 33 mg pigment per Kg of feed produced yolk colour which is considered acceptable to the consumer. With an increase in the amount of pigment in the feed, colour deposition in the organs increased, but efficiency of utilization was lowered.

The fish fed marigold flower experienced yellow pigmentation, which was remarkably different from the other groups. (Buyukcapar *et al.*, 2007) reported that the most appropriate dietary doses of marigold flower and red pepper for pigmentation of rainbow trout are 1.6 percentages and 4.4 percentages respectively.

Goldfish (*Carassius auratus*) are in demand in world markets due to their attractive golden colour. Carotenoids are the primary source of colour in the skin of fish. To optimize the colour in captivity, fish must obtain an adequate level of carotenoids in

their feed. The study shows that natural carotenoid enhance its colour and also accelerate gonadal development (Archana and Ahmed., 2007).

Breithaupt, (2007) reviewed the application of xanthophylls in poultry farming and in aquaculture (trout and salmon), referring to natural (e.g. lutein from *Tagetes erecta*) as well as synthetic (e.g. canthaxanthin) xanthophylls. Additionally, an overview about the legal position in the European Union is given.

Lutein containing functional foods and nutraceutical products has been developed in recent years to help needy populations acquire sufficient lutein intake through supplementation. Lutein is also among the 10 phytochemicals recommended by the FDA as GRAS (generally recognised as safe) nutritional supplements. Recent research efforts have focused on the use of marigold extracts as human nutritional supplements. Hence effects of extraction procedures on the quality and quantity of the extracts have become important.

Materials and methods

Materials and methods

The present investigation on "Extraction, preservation, and utilization of natural colour from marigold (*Tagetus erecta* L.)" was carried out at the Department of Processing Technology, College of Horticulture, Vellanikkara, Thrissur during 2007-2009.

In this study an attempt has been made to standardize the protocol for the separation of natural colour from marigold, to preserve the extract into powder/concentrate, its application in food products and to study the changes during storage.

The whole programm e was divided in to three major experiments

1. Preparation of raw material and extraction of colour
2. Drying and packaging studies
3. Application in food and storage studies

3.1. Preparation of raw materials and extraction

Marigold flowers were purchased from local market, Thrissur. (Plate1). The extraction of colour was done using dried flowers as well as cured flowers**.** Flowers procured were weighed and divided into two batches. One was used for direct extraction and the other for curing. Marigold flowers were purchased from local market, Thrissur. (Plate1). The extraction of colour was done using dried flowers as well as cured flowers**.** and weighed separately in each batch (Fig.1).

3.1.1. Drying

The Petals were dried under ambient conditions for five days during which the temperatures ranged from 28 to 35°C until the moisture content reduced to 10 per cent. The dried petals were ground using a grinder to marigold flower flour of 0.5mm size (Plate 2). Physical properties like initial colour, texture, particle size and moisture content, recovery percentage were recorded.

3.1.1.1. Observations

3.1.1.1.1. Recovery percentage

Weight of sample after drying over initial weight was calculated and expressed as recovery percentage.

Marigold bloom in field

Plant with flowers

Marigold flower

Plate 1

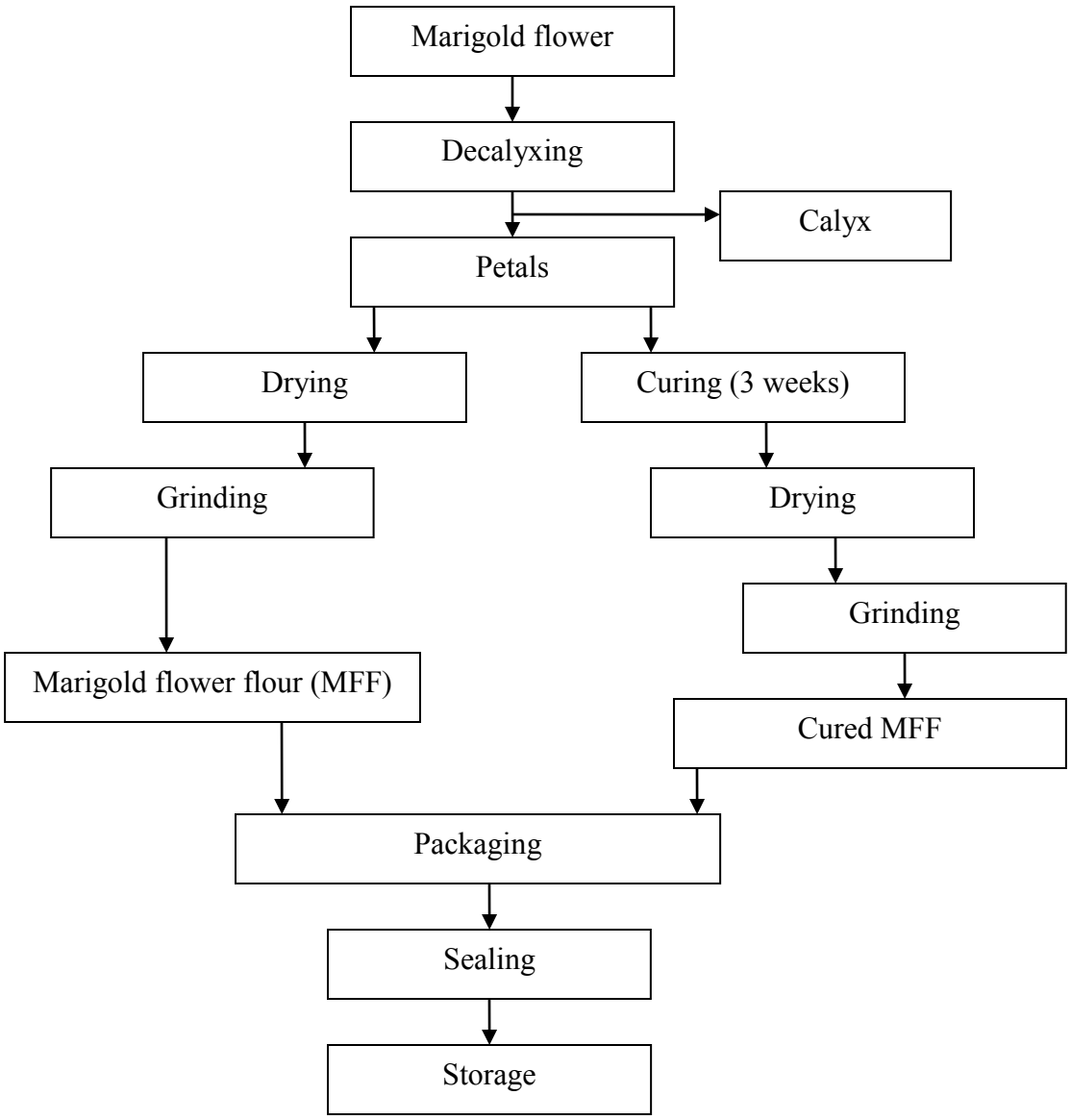
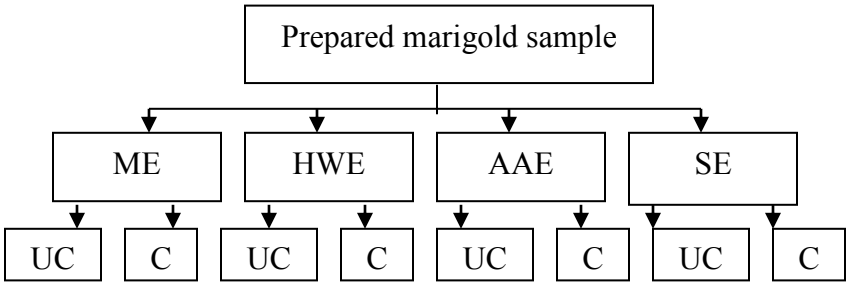


Fig.1. **Preparation of raw material for colour extraction from marigold**



ME - Mechanical extraction, HWE - Hot water extraction, AAE - Acidified aqueous extraction, SE - Solvent extraction, UC - Uncured sample, C - Cured sample

Fig.2. **Methods of colour extraction from marigold**

Preparation of raw material for uncured sample

Marigold flower

Petals

Drier

Shade drying

Dried flower

Uncured flower flour

Plate 2

$$\text{Recovery percentage} = \frac{\text{Final weight after drying}}{\text{Initial weight}} \times 100$$

3.1.1.1.2. Residual moisture

Moisture content was recorded directly using MB 45 moisture analyser, (OHAUS, Switzerland).

3.1.1.1.3. Particle size

Particle size of the dried marigold flower flour was measured using a sieve of 0.5 mm mesh size.

The powder was stored at 4 °C after packing in sealed black polyethylene bag until they were used for experimental assays.

3.1.2. **Curing**

Petals were heaped on a 300 gauge polythene sheet and was covered with 150 gauge polythene sheet and maintained for three weeks in a closed yard under ambient conditions. After three weeks, the product obtained was dried at 60°C in a cabinet drier till the moisture content reduced to 8% (±1%), and ground to obtain fermented marigold flower flour of size 0.5 mm. Initial physical properties like colour, texture, particle size and moisture content using MB 45 moisture analyser (OHAUS, Switzerland) were recorded as mentioned in the case of dried sample (Plate 3).

3.1.3. **Extraction of colour**

Four extraction methods were carried out for both dried and cured flowers (Fig.2 & Plate 4, 5).

1. Mechanical extraction
2. Hot water extraction
3. Acidified aqueous extraction
4. Solvent extraction

3.1.3.1 **Mechanical extraction**

Mechanical extraction was carried out in both fresh flowers as well as cured flowers.100 grams each samples were taken and crushed using a grinder, squeezed it using muslin cloth to get the extract.

Preparation of raw material for cured sample

Marigold flower

Petals

Curing

Cured flower

Drying

Cured dried flower

Cured flower flour

Grinding

Plate 3

Extraction methods for uncured flower flour

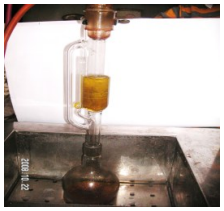
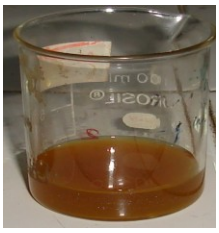
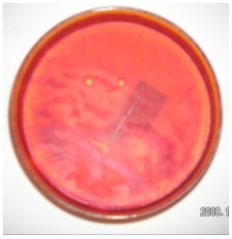
Mechanical extraction	Hot water extraction	Acidified aqueous extraction	Solvent extraction
			
Crushing	Heating	Immersed in acidified water	Soxhlet extraction
⇩	⇩	⇩	⇩
			
Mechanical extract	Hot water extracts	Acidified aqueous extracts	Solvent extract
⇩	⇩	⇩	⇩
			
Dried extract	Dried extract	Dried extract	Oleoresin

Plate 4

Extraction methods for cured flower flour

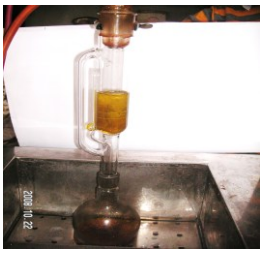
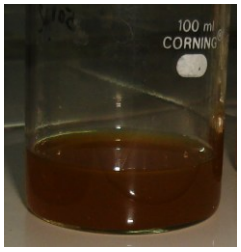
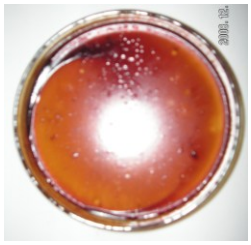
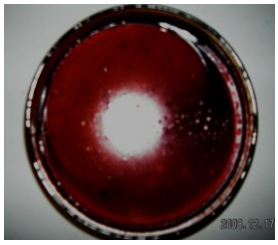
Mechanical extraction	Hot water extraction	Acidified aqueous extraction	Solvent extraction
			
Crushing	Heating	Immersed in acidified water	Soxhlet extraction
⇩	⇩	⇩	⇩
			
Mechanical extract	Hot water extracts	Acidified aqueous extract	Solvent extracts
⇩	⇩	⇩	⇩
			
Dried extract	Dried extract	Dried extract	Oleoresin

Plate 5

3.1.3.2. **Hot water extraction**

Hot water extraction was carried out in both dried and cured samples. Different weights of 1, 2, 3, 4, 5g each were taken in 250 ml conical flasks, added 100 ml of water in each flask, and boiled for two hours in water bath. The extract was filtered through Whatman no.1 filter paper. It was diluted when the concentration of extract was too high. The absorbance of the filtered extract was recorded at different intervals of time 30, 60, 90, 120 minutes at 446 nm using a spectrophotometer (Thermospectronic, Geneses 20, USA). Yield was quantified and xanthophylls concentration was determined as per AOAC, (1984). The process was replicated thrice for both dried as well as cured samples.

3.1.3.3. **Acidified aqueous extraction**

Two grams of samples were taken and soaked in the citric acid solution of different strengths 1, 2, 3, 4 and 5 % (Main *et al*., 1978). The extract was filtered through Whatman no.1 filter paper and absorbance was recorded at different intervals of time 0, 1, 2, 3, 4, 5 and 24 hours using spectrophotometer (Thermospectronic, Geneses 20, USA) at 446 nm using citric acid solution as blank. Yield was quantified and xanthophylls concentration was determined as per AOAC, (1984). The process was replicated thrice both in dried and cured samples.

3.1.3.4. **Solvent extraction**

Oleoresin from flower flour was extracted in a batch process using analytical grade solvents. The extraction was performed in a Soxhlet apparatus composed of 500 ml round bottom flask with three necked top connected to the condenser. The batch extractor was loaded with 5g sample in a filter paper packet. For extraction solvent used was hexane: acetone mixture (7:3) and the content was heated to temperature of 100°C in water bath and continued for a period of 2 hours. Extractions were stopped when saturated solutions were obtained and the solvent turned colourless

Extracts were collected in a petridish and solvent was evaporated by drying in a hot air oven to get free oleoresin. The extraction was replicated thrice for both dried and cured samples. Yield was quantified and xanthophylls concentration was determined as per AOAC, (1984).

Saponification process

Oleoresin

Heating with KOH

Precipitation using $CaCl_2$

Filtration

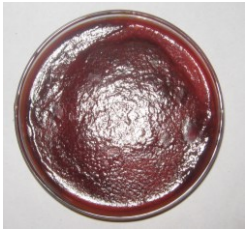
Filtered cake

Drying

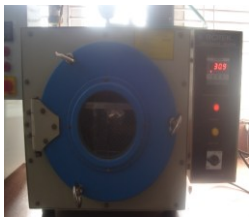
Dried cake

Washing with ethanol

Recovery of ethanol



Marigold colour



Vacuum drying

Dried lutein powder

Plate 6

3.1.4. **Saponification**

The extract containing lutein esters were saponified to get free lutein from the oleoresin (Rosales and Cardona., 2006). (Plate 6 & Fig.3). 1g of extracted oleoresin in a conical flask, 15 ml of 50% KOH solution and 5ml of propylene glycol were added and heated to 110 °C for an hour under moderate agitation. Material thus obtained was dissolved in 4L of water and temperature adjusted to 50°C, when the dispersion was completely homogeneous the pH was reduced to 4 using 5% aqueous calcium chloride solution. An abundant precipitate was formed, containing mainly calcium salts of fatty acids and it was filtered using Whatman no.40 filter paper to remove as much liquid as possible. The resulting cake was dried and washed using two methods,

1. Using 1% solution of calcium chloride in acetone
2. Using ethanol

at room temperature until the liquid runs clear .The solvent was recovered and it was dried in vacuum drier to get free lutein crystals. The crystals were ground to get fine lutein powder, which was again dried in vacuum drier to remove maximum amount of solvent.

3.1.5. Estimation of lutein

Fifteen milli grams of sample was weighed into a 100 ml volumetric flask. The sample was diluted to the mark using solvent mixture (hexane: ethanol: acetone: toluene in the ratio10:6:7:7). The mixture is agitated or stirred until the sample dissolved. One ml of this solution was transferred by pipette into a 100 ml amber coloured volumetric flask and diluted to the mark with ethanol. The absorbance of the ethanol solution was measured at 446 nm using spectrophotometer (Thermospectronic, Geneses 20, USA) that has been calibrated to zero with ethanol.

$$\text{Percentage of xanthophylls as lutein} = 1000A/225W$$

In which A - Absorbance of the test solution

W - Weight in grams of sample taken to prepare the test stock solution

255 - Absorptivity of the pure lutein

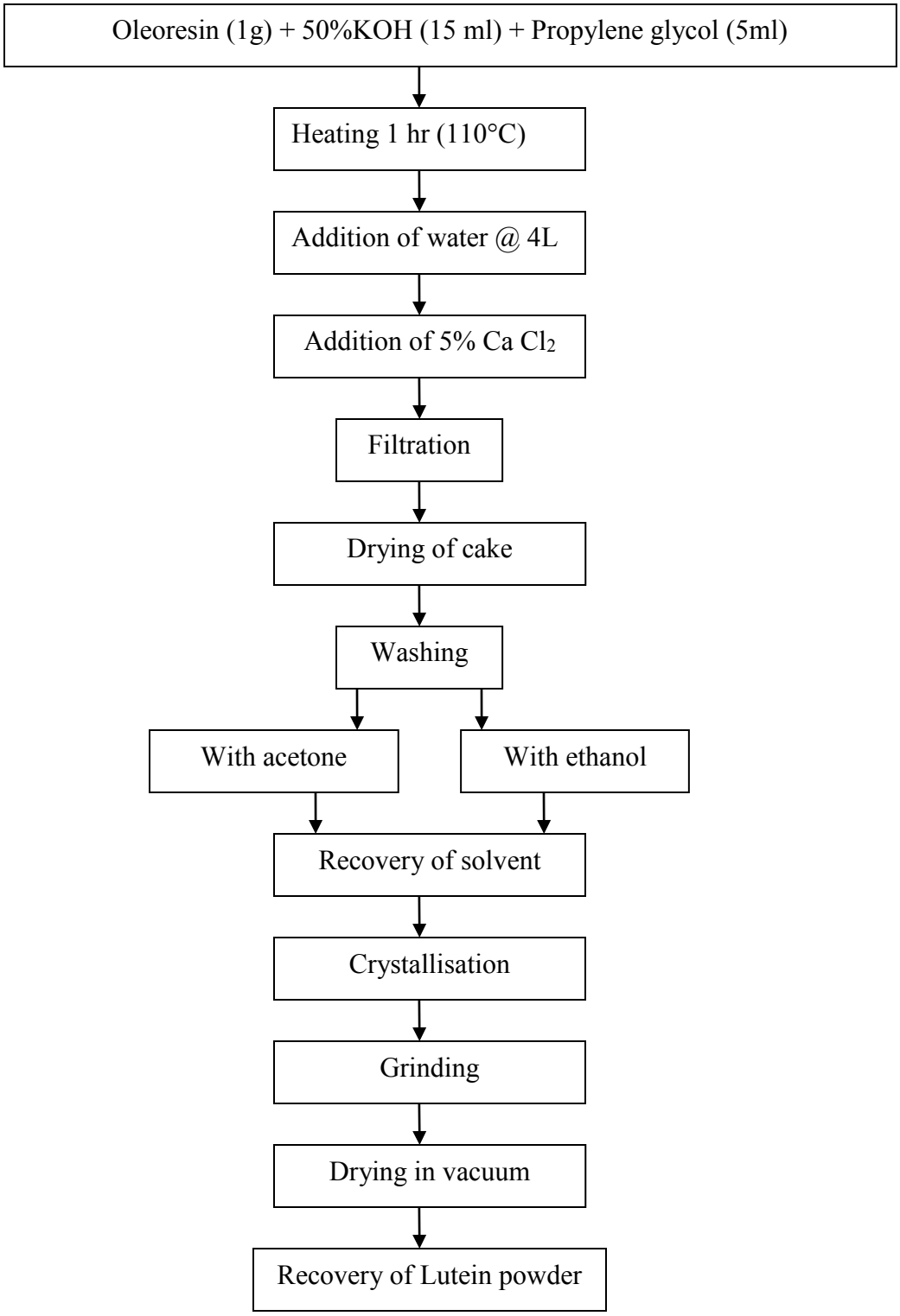


Fig.3 **Saponification of marigold oleoresin**

Gas Chromatographic Mass Spectrometry

Plate 7

Gas chromatographic mass spectrometry (GC-MS) analysis

This test was carried out to detect the volatile solvents present in the crystalline lutein sample as well as in the lutein formulation.

The instrument consists of a gas chromatograph with a quadrupole mass spectrometer (MS) as a detector (Plate 7). The sample consisting of a mixture of organic compounds is introduced to the head of the column. Gas inside serve as carrier to move the sample through the column, where the mixture is separated into individual components. As each component issues from the column it is introduced in to the mass spectrometer where a mass spectrum for that material is obtained and stored in a computer. Based on the mass spectrum the compound is identified. Qualitative and quantitative analysis of the residual solvent present in the crystalline lutein powder was made using GC-MS test. From the graph obtained the concentration of the residual solvent was calculated using the formula

$$\text{ppm of solvent} = \frac{\text{Area of the solvent}}{\text{Area of IS}} \times \frac{\text{weight of IS}}{\text{Weight of sample}} \times \text{ppm of IS} \times \text{CF}$$

IS - Injection solvent

CF - Correction factor

3.2. Drying and packaging

The marigold lutein crystal obtained after saponification was dried in a vacuum drier (Fortech microprocessor, Rotek, India) at a temperature of 60°C twice or thrice to remove the solvent residue. It was then packed in glass bottles and stored at 4°C for further studies.

3.2.1. Utilisation of colour

The purified lutein powder obtained was used for the preparation of a formulation, which is convenient for the consumer to use in the food product directly.

3.2.1.1. Preparation of lutein formulation

The lutein crystals obtained is a fat soluble pigment. It was made to a paste using vegetable oil containing 80% refined soyabean oil and 20% refined sunflower oil. Formulations with different concentrations at 1:10, 1:5, 1:2 (lutein: oil) were prepared and filled in glass vials and stored at room temperature. Solvent residue

of the formulations were tested using gas chromatography (GC-MS) for its utilisation as food colourant.

The best formulation was identified on the basis of high intensity of colour, low amount of oil, easiness of preparation, and utilised for the application and storage studies

Cost of production

Cost of production of 100 g finished product was worked out based on the cost of inputs, overhead and utilities. Working capital included the cost of raw materials viz., marigold, chemicals, cost of fuel, and labour involved for the production of 100 g of lutein formulation.

3.3 Application and storage studies

The lutein formulation was added to different food products like ice cream, yogurt, oil, juice and milk and standardised the quantity of the formulation to be added to get an appropriate colour to each product by sensory evaluation on a five point hedonic scale involving trained panel. The initial sensory qualities like colour, flavour and overall acceptability was studied in comparison to the respective products added on a five point hedonic scale involving trained panel. The coloured products were stored and periodical estimation of residual colour pigments in combination with food products and the changes with respect to temperature, light and pH were compared with that of synthetic food colours.

Organoleptic evaluation

Organoleptic scores for colour, flavour, taste, consistency and overall acceptability of the natural coloured product were recorded over a five point hedonic scale involving trained panel (Amerine *et al*., 1965). Score cards used in the evaluation is given in the Appendix.

3.3.1**. Application in food products**

3.3.1.1. Application in ice-cream and yogurt

Four samples each of 50 g of ice cream and yoghurt were taken. The best colour formulation prepared using 1:2 lutein: vegetable oil was added in different concentrations T_1, T_2, T_3 (1, 2, 3, drops respectively) for each sample. A blank T_4 was kept with out adding any colour. The best concentration of colour which gives the most appealing colour was identified through sensory evaluation. The initial absorbance of the colour eluted from each sample of ice cream and yoghurt using

ethanol was recorded. The products kept for storage at - 18°C and the absorbance was recorded at weekly intervals.

3.3.1.2. Application in juice

Four samples each of 50 ml lemon juice were taken in beaker. The best colour formulation prepared using 1:2 lutein: vegetable oil was added in different concentrations T_1, T_2, T_3 (1, 2, 3, drops respectively) each sample. A blank T_4 was kept with out adding any colour.

3.3.1.3. Application in milk

Four samples each of 50 ml milk were taken in beaker. The best colour formulation prepared using 1:2 lutein: vegetable oil was added in different concentrations T_1, T_2, T_3 (1, 2, 3, drops respectively) each sample. A blank T_4 was kept with out adding any colour.

3.3.1.4. Application in oil

Four samples of 50 ml of coconut oil were taken. The best colour formulation prepared using 1:2 lutein: vegetable oil was added in different concentrations T_1, T_2, T_3 (1, 2, 3, drops respectively) each sample. A blank T_4 was kept with out adding any colour. The best concentration of colour which gives the most appealing colour was identified through sensory evaluation.

3.3.2. Storage studies

Storage studies were conducted to confirm the stability of the natural colour in the product on storage as well as the changes occurring due to the effect of temperature, light and pH in comparison with synthetic colours during storage.

3.3.2.1. Changes due to storage

The best sample of natural coloured ice cream and yoghurt identified through sensory evaluation was selected. Matching coloured samples of ice cream and yoghurt were also prepared using 10 mg each lemon yellow and orange red synthetic colour formulations. The initial absorbance of the colour eluted from each sample using ethanol was recorded. Then it was stored at - 18°C to study the changes during storage. Absorbance was recorded at weekly intervals.

3.3.2.2. Changes due to light

The best sample of oil identified through sensory evaluation was selected. A matching coloured oil samples was also prepared using 10 mg orange red synthetic colour formulation. Both of them were stored in transparent glass bottle and amber coloured bottles and kept under light at room temperature. Blank samples were also

kept with out adding any colour. The absorbance of the colour eluted from the sample using ethanol was recorded initially and at weekly intervals.

3.3.2.3 Changes due to heat

Fifty milli litters of the best sample of natural coloured oil identified through sensory evaluation was taken in a 100 ml conical flask. Matching oil samples was prepared using 10mg orange red synthetic colour formulation and taken in another conical flask. Blank sample were also kept with out adding any colour. The samples were heated at boiling temperature for 30 minutes. The absorbance of the colour eluted from the sample using ethanol was recorded initially and at 10 minutes intervals.

3.3.2.4. Changes due pH

The initial pH and the pH after eight weeks of storage of natural coloured as well as the synthetic coloured ice cream and yoghurt were measured using pH analyser, LI 612 (ELICO, India) which was calibrated before use, with reference solutions pH 4.0 and pH 7.0 buffers. Measurements were taken at 22°C by inserting the electrode directly into the samples.

3.3.2.5. Comparison of marigold lutein with synthetic colours

3.3.2.5.1. Ice cream

Three samples each of 50g ice cream were taken. Two drops of MG (marigold colour formulation1:2), 10mg each of LY (lemon yellow colour), and OR (orange red colour) were added in each sample. The characters like initial colour, flavour and overall acceptability of the samples were compared. The best sample which gave the most appealing colour was identified through sensory evaluation on a hedonic scale involving trained panel. The product stored at -18°C and the changes in colour, flavour, consistency and taste were noted at weekly intervals. In each week the stored samples were compared with freshly prepared samples. The absorbance of the stored samples was also recorded in each week.

3.3.2.5.2. Yoghurt

Three samples each of 50g yoghurt were taken. Two drops of MG (marigold colour formulation1:2), 10mg each of LY (lemon yellow colour), and OR (orange red colour) were added in each sample. The characters like initial colour, flavour and overall acceptability of the samples were compared. The best sample which gave the most appealing colour was identified through sensory evaluation on a hedonic scale involving trained panel. The product stored at -18°C and the changes in

colour, flavour, consistency, and taste was noted at weekly intervals. In each week the stored samples were compared with freshly prepared samples. The absorbance of the stored samples was also recorded in each week.

Statistical analysis of data

Data were analysed as a completely randomised design (CRD) for all the parameters using SPSS statistical software. Significant difference between means was estimated using Kendall's Wall test. Data pertaining to organoleptic evaluation were analysed using Mann-Whitney test.

Results

Results

The results of the study extraction, preservation and utilization of natural colour from marigold are presented under the following headings.

1. Preparation of raw materials and extraction of colour
2. Drying and packaging studies for colour
3. Application of colour in food and storage studies

4.1. **Preparation of raw materials and extraction of colour**

4.1.1. **Preparation of raw materials**

Petals were separated from the whole flower. The recovery of petals obtained from 10Kg flower was 6.25Kg (62.5%) (Fig. 4, 5). In order to standardise the best extraction method suitable for the maximum extraction of colour from marigold flower, cured and uncured flowers were used as raw materials (Plate 8).

4.1.1.1. **Raw material recovery**

Percentage recovery of the marigold flower flour from cured and uncured samples was different. In the case of uncured samples the mean percentage recovery was found to be 7% and that of cured samples was 3% (Fig. 5, 6).

4.1.1.2. **Physical characters**

The physical characters like colour, texture, odour, particle size and moisture content of the prepared raw materials (cured and uncured) are presented in Table 1.

4.1.1.2.1. **Colour**

The uncured flower flour obtained after drying and grinding process was orange in colour whereas in cured samples it was blackish brown in colour.

4.1.1.2.2. **Texture**

In both cases of cured and uncured samples the texture of the flower floor was found to be course powder in nature.

4.1.1.2.3. **Odour**

The uncured sample has got a characteristic marigold odour while the cured sample has got a peculiar fermented flower odour.

4.1.1.2.4. **Particle size**

Particle size of both cured and uncured samples were found to be same of 0.5 mm each.

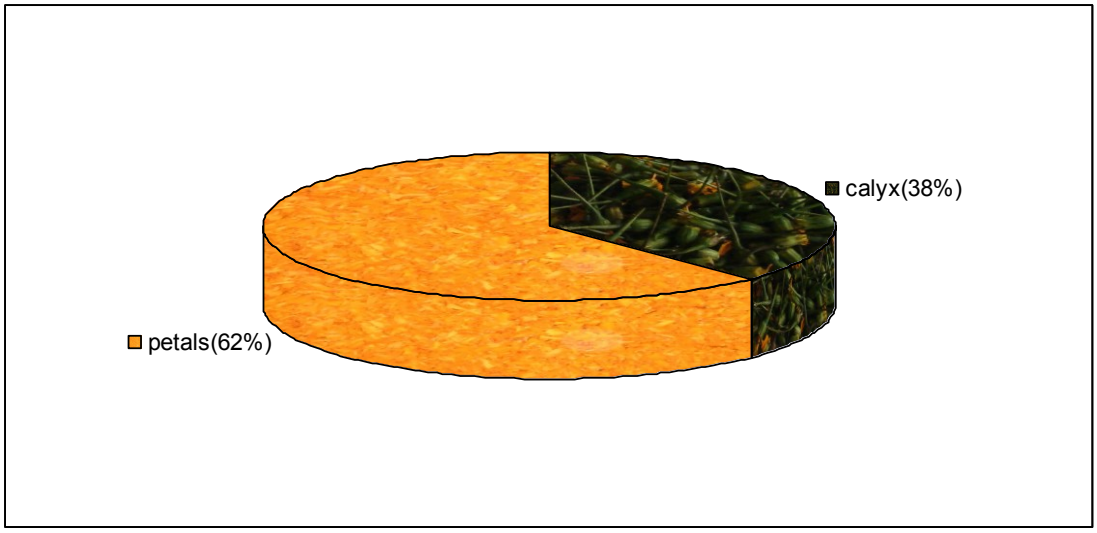


Fig.4. Percentage recovery of petal and calyx from marigold flower.

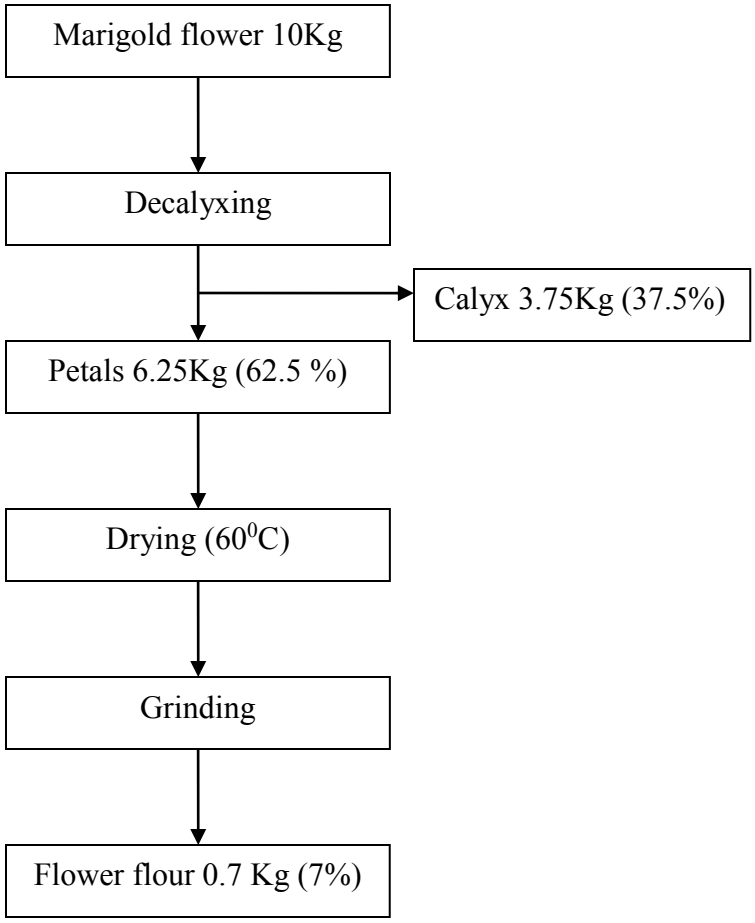


Fig . 5. Percentage recovery of marigold flower flour from uncured sample

Preparation of raw materials

Marigold flower petals

Shade drying

Curing

Dried flower flour

Cured flower flour

Plate 8

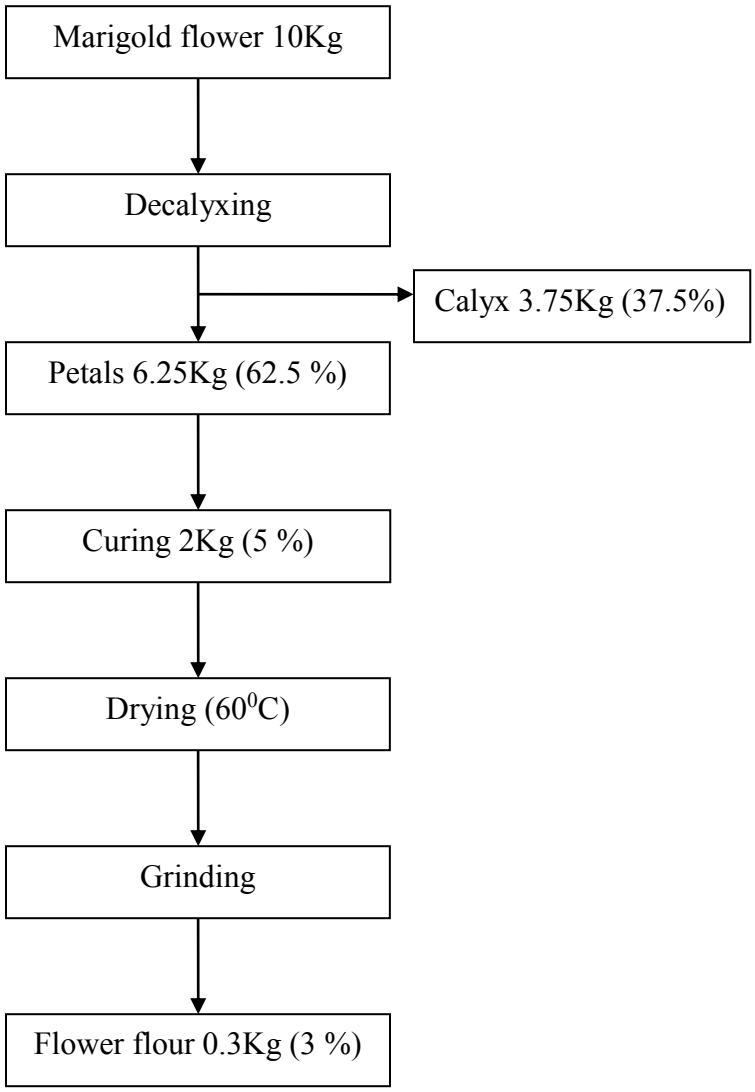


Fig.6. Percentage recovery of marigold flower from cured sample

Table 1. Physical characters of cured and uncured flower flour

Sample	Colour	Texture	Odour	Particle size (mm)	%Moisture*
Uncured	Orange	Coarse powder	Characteristic marigold odour	0.5	10.47
Cured	Blackish brown	Coarse powder	Fermented flower odour	0.5	8.86

* Mean value replications

Table 2. Recovery and characters of mechanical extract of cured and uncured samples

Samples	% Recovery*		Characters of extract	SE	
	Extract	Lutein		Extract	Lutein
Uncured	30	1.03	Dull orange, turbid fluid	0.33	0.005
Cured	24	1.53	Brown coloured turbid fluid	0.33	0.011

*Mean value replications
SE- Standard error

4.1.1.2.5. **Moisture content**

Moisture content of both the samples measured at 170^{o}C using MB 45 moisture analyser (OHAUS, Switzerland) recorded percentage moisture of 10.47 for uncured samples and 8.86 for cured sample.

4.1.2. **Extraction of colour**

Four extraction methods viz. mechanical extraction, hot water extraction, acidified aqueous extraction, solvent extraction were carried out for both dried and cured flowers

4.1.2.1. **Mechanical extraction**

The absorbance of the extract, both from fresh and cured flowers were recorded at 446 nm using spectrophotometer. The extract obtained from fresh flowers was dull orange, turbid fluid. It contained very low amount of lutein. Recovery of extract was 30% and lutein 1.03%. In cured samples the extract obtained was brown coloured turbid fluid. Recovery of extract was 24 % and lutein was 1.53%. Percentage recovery of lutein was higher in cured sample compared to uncured sample. Table 2.

4.1.2.2. **Hot water extraction**

The absorbance of the filtered extract of hot water extraction recorded at different intervals of time 30, 60, 90, 120 minutes at 446 nm using spectrophotometer showed that, in all the treatments absorbance increased with time of boiling form 0 h to 2 h. Maximum absorbance was recorded when the samples extracted for 2 h. After 2 h there was not much increase in absorbance. Percentage recovery of the extract, lutein and the characters of the extract recorded in both uncured and cured samples are given in Table 3.

4.1.2.1.1. **Uncured sample**

The extract obtained was light orange in colour with turbid consistency. The extract contained very low amount of lutein. Recovery of extract was 21% and lutein 1.86%.

4.1.2.1.2. **Cured sample**

In cured samples the extract obtained was dull black in colour, turbid consistency with high amount of impurities. Recovery of extract was 25 % and lutein was 3.33%. Percentage recovery of extract as well as lutein was higher in cured sample compared to uncured sample.

4.1.2.3. **Acidified aqueous extraction**

The absorbance of acidified aqueous extract recorded at different intervals of time 0, 1, 2, 3, 4, 5, and 24 h using spectrophotometer showed that, in both cured and uncured samples absorbance increased with increase in concentration of citric acid up to 4% and found decreasing when the concentration of citric acid increased above 4%. Absorbance also increased with increased time upto 4 h, afterwards it decreased. Maximum absorbance was recorded for samples treated with 4% concentration of citric acid for 4 h. Percentage recovery of the extract, lutein and the characters of the extract recorded in both uncured and cured samples are presented in Table 4.

4.1.2.2.1. **Uncured sample**

The brown coloured extract contained very low amount of lutein and large sum of impurities. Recovery of extract was 10.98% and lutein 5.4%.

4.1.2.2.2. **Cured sample**

In cured sample the extract was blackish brown in colour with a recovery of 7% and lutein 18.5%. Percentage recovery of extract as well as lutein was higher in cured sample compared to uncured sample. But the extract contained high amount of impurities.

4.1.2.3.4. **Solvent extraction**

Absorbance of the extract from the uncured and cured flower flour in a batch process using analytical grade solvents, hexane acetone mixture (7:3) taken at different time intervals during the extraction showed an increased absorbance with time till the solvent run colourless. The percentage recovery of oleoresin and lutein was more in cured sample compared to uncured sample (Table 5).

4.1.2.3.1. **Uncured**

In uncured sample the oleoresin obtained after the extraction was an orange-red coloured, water insoluble, Sticky fluid with characteristic marigold odour with recovery of oleoresin 10.12% and lutein 22.86%.

4.1.2.3.2. **Cured**

Oleoresin obtained from cured sample was a dark red coloured, water insoluble, sticky fluid with characteristic marigold odour; with recovery of oleoresin 30.2% and lutein 64.26%.

Table 3. Recovery and characters of hot water extract of cured and uncured samples

Samples	% Recovery*		Characters of extract	SE	
	Extract	Lutein		Extract	Lutein
Uncured	21	1.86	Light orange colour, turbid consistency	0.57	0.005
Cured	25	3.33	Dull black colour and, turbid consistency	_	0.005.

* Mean value replications
SE- Standard error

Table 4. Recovery and characters of acidified aqueous extract of cured and uncured samples

Samples	% Recovery*		Characters of extract	SE	
	Extract	Lutein		Extract	Lutein
Uncured	10.98	5.40	Brown coloured fluid	0.011	_
Cured	7.00	18.50	Blackish brown fluid	0.088	0.014

*Mean value replication
SE- Standard error

4.1.2.3.3. **Comparison of different extraction methods of cured and uncured samples**

The four extraction methods carried out were compared for both cured and uncured samples to find out the best method of extraction (Table 6).

4.1.2.3.3.1. **Recovery**

In both cured and uncured samples solvent extraction recorded highest recovery of extract as well as lutein. In brief solvent extracted cured sample recorded the highest percentage in recovery of extract as well as lutein in all the four methods of extraction. Percentage recovery of extract was higher for the cured sample in mechanical extraction, hot water extraction and solvent extraction. In mechanical extraction and acidified aqueous extraction uncured sample recorded higher recovery of extract. Lutein percentage was higher in cured sample in all the extraction methods

4.1.2.3.3.2. **Characters**

Extracts from hot water extraction and acidified aqueous extraction were black coloured turbid fluids with high amount of impurity. But reddish orange coloured, sticky water insoluble oleoresin with characteristic marigold odour was obtained from solvent extraction.

Cured sample recorded high lutein content as well as oleoresin recovery compared to uncured sample in solvent extraction, which is further purified to get pure lutein crystals.

4.1.3. Saponification

Saponification using potassium hydroxide converted the ester from of lutein present in the extracted oleoresin by solvent extraction method to free lutein. The saponified cake obtained was dried, powdered and further washed using acetone or ethanol to get pure lutein crystals.

4.1.4. Purification of saponified cake

Dried powder obtained through saponification process was further washed using solvents acetone or ethanol to remove the impurities and to get the pure lutein crystals.

4.1.4.1. Acetone

The colour obtained after filtration and evaporation of acetone washed saponified cake was recrystallised by drying in vacuum drier to get lutein crystal. This was ground to get the dark orange coloured fine powder of lutein.

Table 5. Recovery and characters of solvent extract of cured and uncured samples

Samples	% Recovery*		Characters of extract	SE	
	Extract	Lutein		Extract	Lutein
Uncured	10.12	22.86	Orange-red, water insoluble, Sticky fluid with characteristic marigold odour	0.006	0.012
Cured	30.20	64.26	Dark red, water insoluble, sticky fluid with characteristic marigold odour.	0.437	0.655

*Mean value replications
SE- Standard error

Table 6. Comparison of different extraction methods of cured and uncured samples

Method of extraction	Uncured sample			Cured sample		
	% Recovery*		Characters	% Recovery*		Characters
	Extract	Lutein		Extract	Lutein	
Mechanical extraction	30	1.03	Dull orange, turbid fluid	24	1.53	Brown coloured turbid fluid
Hot water extraction	21	1.86	Light orange, turbid	25	3.33	Dirty black, turbid
Acidified aqueous extraction	10.98	5.4	Brown coloured fluid	7	18.5	. Blackish brown fluid
Solvent extraction	10.12	22.86	Orange-red, water insoluble, Sticky fluid with characteristic marigold odour	30.2	64.262	Dark red, water insoluble, sticky fluid with characteristic marigold odour

*Mean value replications

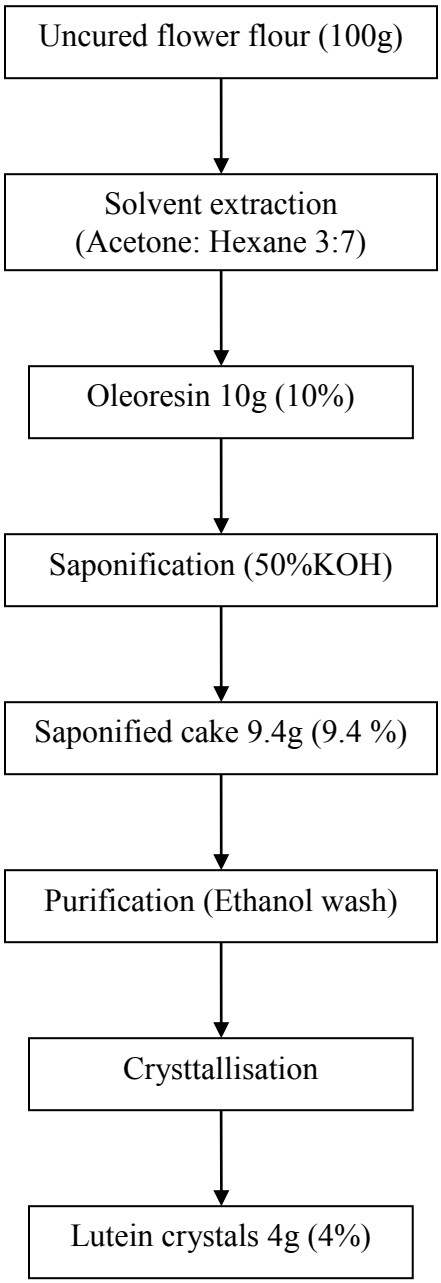


Fig.7. Percentage recovery of lutein from uncured sample

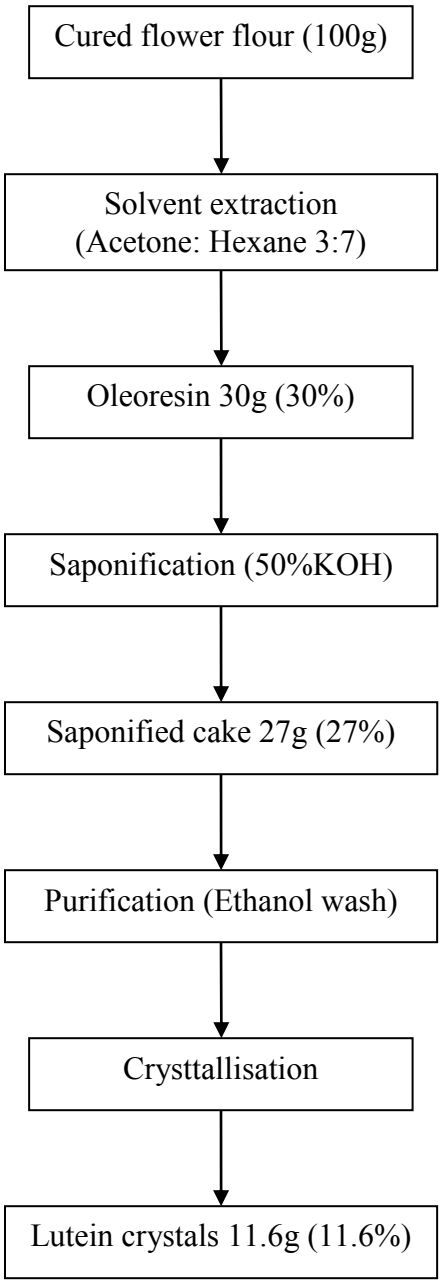


Fig. 8. Percentage recovery of lutein from cured sample

4.1.4.2. Ethanol

The colour ethanol washed cake was further ground to get the dark orange coloured fine powder of lutein. Percentage recovery of lutein after purification from cured and uncured samples was 11.6% and 4% respectively (Fig. 7, 8).

Estimation of lutein

The percentage of purified lutein powder was calculated using the absorbance recorded in the spectrometer. The lutein powder contained 92 percent of xanthophyll as lutein.

Solvent residue in lutein powder

Lutein crystals produced from both acetone washed cake as well as ethanol washed cake was tested for solvent residue using GCMS test (Fig. 9 &10). The result showed that the crystals obtained from acetone washed cake contained 11.4 ppm of hexane and high amount (522.9 ppm), of acetone which is much above the permitted level of 30 ppm in food products. But the ethanol washed cake contained only 10 ppm hexane, and 62.63 ppm acetone. Thus the ethanol washed lutein crystal is better compared to acetone washed lutein (Table 7).

4.2. Drying and packaging

The lutein powder obtained after saponification and recrystallisation was ground to fine powder and dried in a vacuum drier at a temperature of 60°C twice or thrice to remove maximum amount of residual solvent and then packed in glass bottles and stored at 4°C for further studies and the results are given below.

4.2.1. Lutein formulation

lutein formulation in an solution using vegetable oil containing 80% refined soyabean oil and 20 percent refined sunflower oil at different concentrations of 1:10, 1:5, 1:2 (lutein: oil) was prepared (Plate 9 & Table 8). Formulation with lutein: oil concentration 1:2 was found to be the best because of its high concentration of lutein powder, low concentration of oil and colourability, and consistency.

Cost of production

Cost of production of 100g lutein formulation is presented in the Table 9.

Cost of production of 100g formulation worked out to be Rs. 4600.

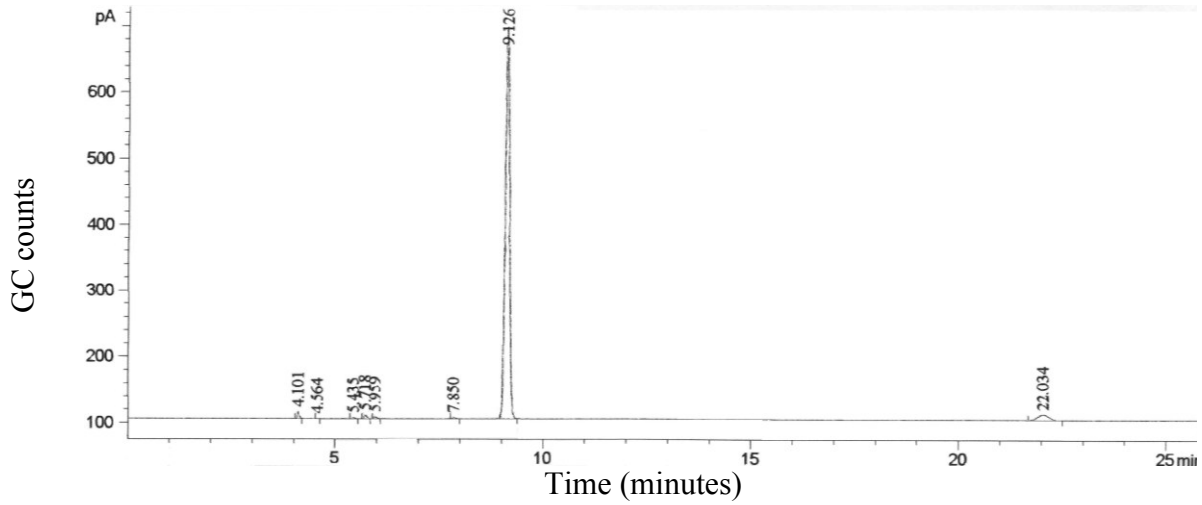


Fig.9. GC-Ms of acetone washed lutein powder

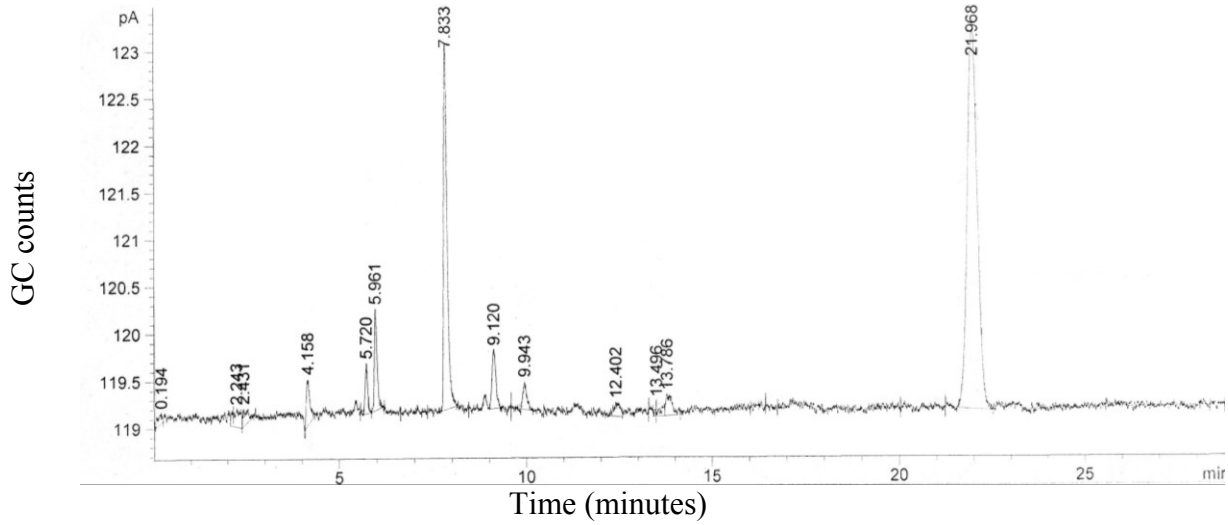


Fig .10. GC-MS of ethanol washed lutein powder

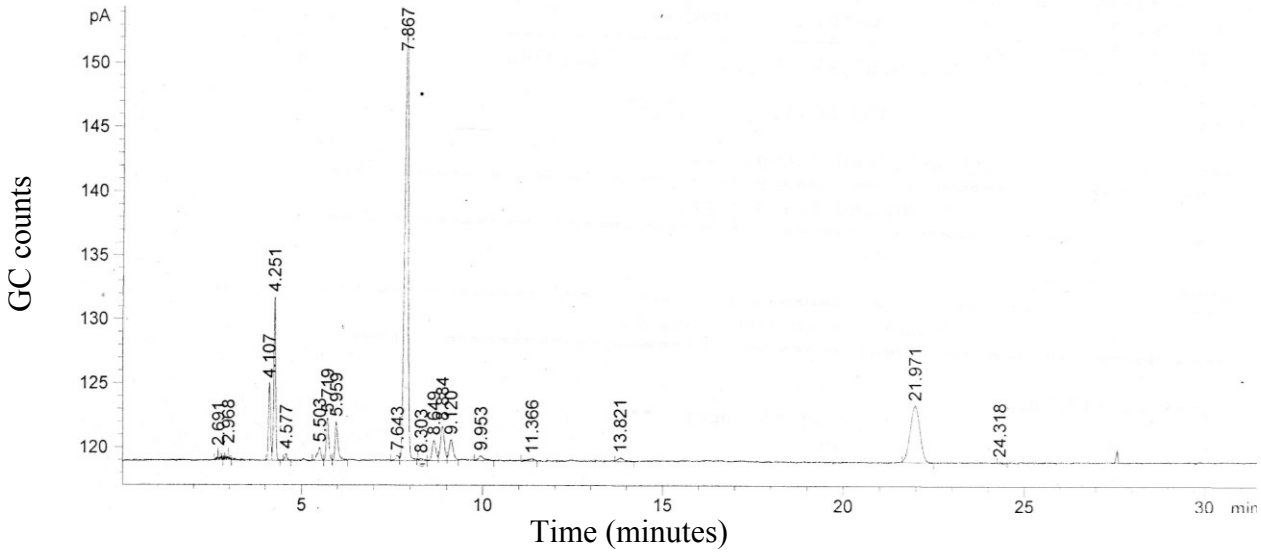


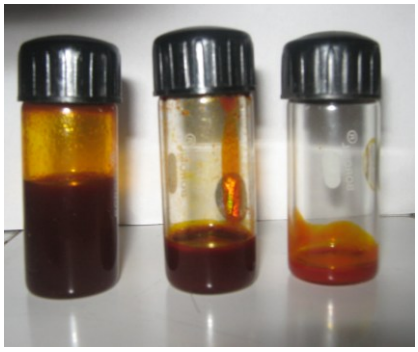
Fig .11. GC-MS of ethanol washed lutein formulation

Preparation of Lutein formulation

Lutein powder

Lutein + soyabean oil

Lutein oil solution (1:2)



(1:10) (1:5) (1:2)

Different lutein formulations

Plate 9

Table 7. **Residual solvent content of purified lutein products**

Product	Solvent residue (ppm)*		
	Hexane	**Acetone**	**Ethanol**
Purified lutein powder (Acetone washed)	11.40	522.90	0
Purified lutein powder (Ethanol washed)	10.00	62.63	145
Lutein formulation	1.70	6.00	144

*Mean value replications

Table 8. **Different Lutein formulations with oil**

Formulation	**Quantity of lutein powder(g)**	**Quantity of oil (ml)**	**Concentration of lutein/drop in gram (1drop=0.1ml)**	**Characters**
1:10	1	10	0.01	Orange red emulsion
1:5	1	5	0.02	Orange red paste
1:2	1	2	0.05	Dark orange red thick paste

Table 9. **Cost of production of100g lutein formulation**

Sl. No	Item	Quantity	Unit cost (Rs.)	Total cost (Rs.)
1	**Raw material**			
	Marigold flower	14.30 Kg	50.00	715.00
	Soyabean oil	100	70.00	7.00
2	**Chemicals**			
	Hexane	3.75 L	406.00	1534.00
	Acetone	1.62L	456.00	738.50
	Calcium chloride	33.75g	216.00	7.29
	Propylene glycol	0.67Kg	522.00	352.00
	Potassium hydroxide	1kg	426.00	426.00
	Ethanol	364.50 ml	1834.00	668.50
3	**Others**			
	Fuel			19.50
	Miscellaneous			120
	Cost of production			**4600.00**

4.2.2. Solvent residue in lutein formulation

Solvent residue of the prepared formulation was tested using gas chromatography (GC-MS) for its utilisation as food colourant (Fig.8). The result showed that all the solvents (hexane- 1.7 ppm, acetone- 6 ppm ethanol- 144 ppm) used in the extraction and purification processes were much below the maximum permitted levels(Table7&Fig. 11).

4.3. Application and storage studies

4.3.1. Application in food

The best formulation found out was added to different food products like ice cream, yogurt, oil, juice and milk and standardised the quantity of the formulation to be added to get an appropriate colour to each product by sensory evaluation on a five point hedonic scale involving trained panel. The results are given in Tables 10 to 12. The natural colouration by marigold was found to be most appropriate for ice cream, yoghurt, and oil (Plate 10). The lutein formulation was not suitable to use in juice and milk as the products contained more amount of water.

Amoung the four concentration levels namely T_1, T_2, T_3 (1, 2, 3, drops of the prepared 1:2 lutein: vegetable oil) tried in ice cream yoghurt and oil, Kendal's Wall test for the three products separately revealed that T_2 (2 drops) was most appropriate for ice cream and yoghurt, where as T_3 (3 drops) was found to be the best for oil.

4.3.2. Storage studies

Stability of lutein

Stability of lutein in fortified products during storage is an important aspect to make the final product attractive and acceptable. Degradation of lutein not only affects the attractive colour of foods but also their nutritive value, taste, flavour, texture and consistency too. Therefore, the stability of extracted pigments in the products during storage was studied in comparison to synthetic colour and the changes with respect to temperature, light, and pH, were also carried out using UV spectrophotometer. The results are given below

4.3.2.1. Changes due to storage

The natural and synthetic coloured ice cream and yoghurt were kept for storage in refrigerated conditions at - 18°C. The initial absorbance of the eluted colour from the products was recorded. And the absorbance was recorded at weekly intervals

Table 10. **Score for sensory attributes of natural coloured ice cream using different quantity of lutein formulation (1:2)**

Lutein concentrations	Mean rank			
	Colour	Flavour	Taste	Consistency
T1	3.00	2.00	2.00	2.30
T2	3.70	3.20	3.30	3.50
T3	1.40	2.40	2.30	2.30
T0	1.90	2.40	2.40	1.90
%Significance*	0.7	22.3	15.6	5.2

Table 11. **Score for sensory attributes of natural coloured yoghurt using different quantity of lutein formulation (1:2)**

Lutein concentrations	Mean rank			
	Colour	Flavour	Taste	Consistency
T1	2.90	2.80	2.30	2.50
T2	3.60	2.80	3.70	2.90
T3	2.10	2.00	1.40	2.50
T0	1.40	2.40	2.60	2.10
%Significance*	1.6	46.8	1.8	57.2

Table 12. **Score for sensory attributes of natural coloured oil using different quantity of lutein formulation (1:2)**

Lutein concentrations	Mean rank			
	Colour	Flavour	Taste	Consistency
T1	2.00	2.80	2.00	2.20
T2	2.80	2.00	2.40	2.60
T3	3.60	3.20	2.80	2.60
T0	1.60	2.00	2.80	2.60
%Significance*	1.5	19	39.2	87.5

T1 0.01ml of 1:2 lutein formulation
T2 0.02ml of 1:2 lutein formulation
T3 0.03ml of 1:2 lutein formulation
T0 Control

Values are mean rank score based on Kendall's W Test
* Significance level fixed at 20%

during the entire eight weeks of storage period, and the results are presented in Table13 & Fig. 12.

In the case of ice cream, it was found that initially there was no significant change in colour up to 4th week of storage in all the samples. Subsequently a decrease in absorbance in the natural coloured sample was observed showing the degradation of marigold colour; where as in the synthetic coloured samples no significant changes were observed till the end of the storage (Table 13 & Fig.13).

In yoghurt also no significant change was found up to the second week of storage, afterwards an increased reduction in absorbance was observed showing the degradation of colour during the remaining period, while in the synthetic coloured samples no significant changes in the absorbance was observed during the entire eight period of storage (Table 13 & Fig. 14).

4.3.2.2. Changes due to light

The absorbance of the colour eluted from natural and synthetic coloured coconut oil the samples stored in transparent glass bottles and amber coloured bottles kept under light at room temperature was recorded initially and at weekly intervals. Results are shown in Table 14 & Fig. 15.

The result showed that absorbance values decreased significantly both in amber coloured and transparent bottles during the entire period of storage. Significant degradation in colour was initiated from the first week itself and from second week onwards a steady decrease was observed with more degradation observed in transparent bottle than that of in the amber coloured bottle. Where as the synthetic coloured product, whether in amber or transparent bottle doesn't have any significant change during the entire period of storage (Plate 11).

4.3.2.3. Changes due to heat

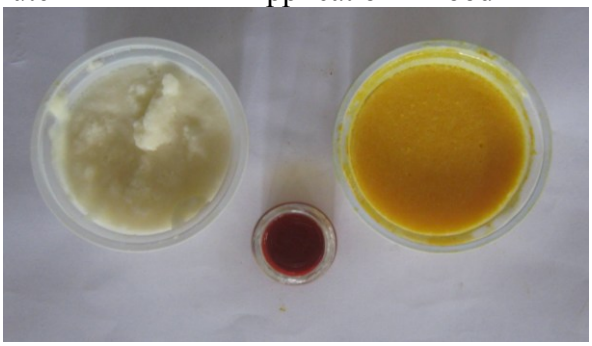
Exposure to thermal treatment resulted in degradation and isomerization of the carotenoids, like beta carotene and lutein. The stability of natural and synthetic colour was studied by heating100 ml each of natural and synthetic coloured oil samples.

The absorbance of the colour eluted from the coloured oil sample using ethanol recorded initially and at 10 minutes intervals are presented in Table 15 & Fig.16. From the result the degradation of colour in natural coloured oil due to heat is evident. Colour decreased fast on heating showing the instability of natural colour on heating compared to synthetic colour (Plate11).

Application of lutein formulation in food products

Lutein

Application in food



Ice cream before adding colour

Coloured ice cream

Yoghurt before adding colour

Coloured yoghurt

Oil before adding colour

Coloured oil

Plate 10

Table 13. **Mean weekly absorbance of synthetic and natural coloured ice cream and yoghurt on storage**

Storage period in weeks	Absorbance at 446nm *			
	Ice cream		Yoghurt	
	Marigold colour	Synthetic	Marigold colour	Synthetic
week1	0.355	0.358	0.561	0.357
Week2	0.352	0.357	0.545	0.357
Week3	0.351	0.356	0.526	0.356
Week4	0.348	0.355	0.504	0.348
Week5	0.34	0.347	0.487	0.339
Week6	0.324	0.339	0.483	0.338
Week7	0.295	0.338	0.471	0.332
Week8	0.273	0.332	0.42	0.325

*Mean value replication

Table 14. **Mean weekly absorbance of synthetic and natural coloured oil on storage under light**

Storage period in weeks	Absorbance at 446nm *			
	Marigold colour		Orange red	
	Amber bottle	Transparent bottle	Amber bottle	Transparent bottle
week1	0.0170	0.0170	0.0115	0.0115
Week2	0.0123	0.0133	0.0115	0.0114
Week3	0.0116	0.0130	0.0113	0.0112
Week4	0.0100	0.0113	0.0114	0.0108
Week5	0.0070	0.0106	0.0112	0.0106
Week6	0.0023	0.0090	0.0112	0.0104

*Mean value replication

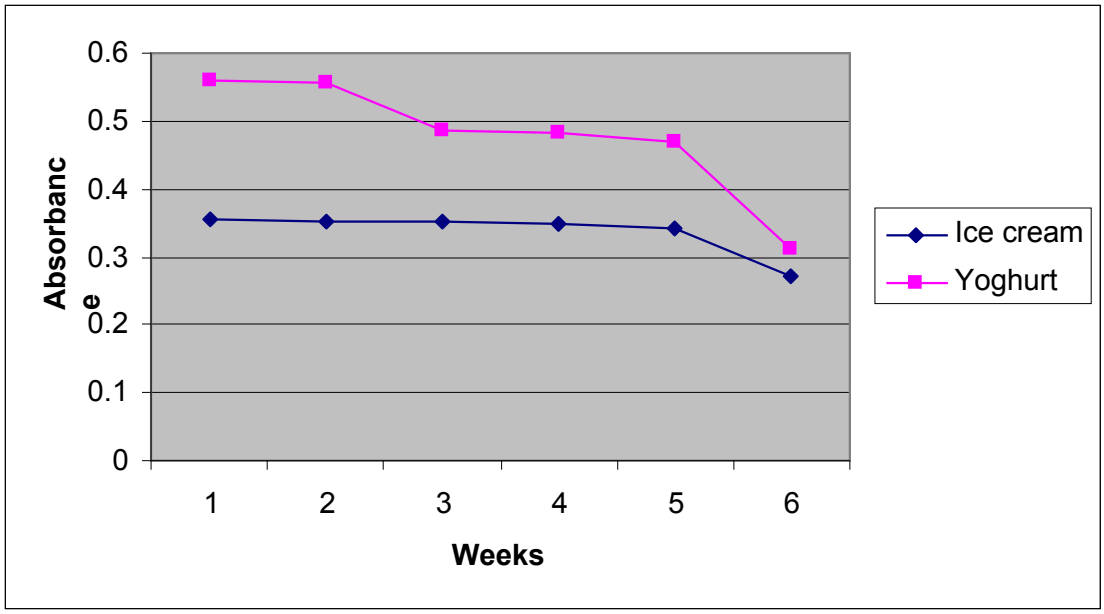


Fig.12. Effect of storage on colour of coloured ice cream and yoghurt

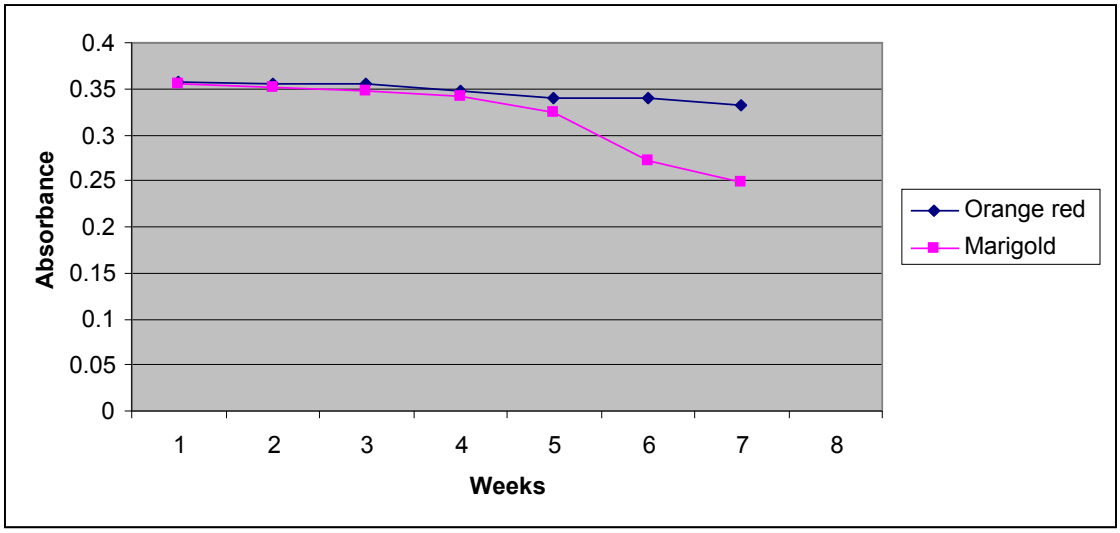


Fig.13. Effect of storage on colour of natural and synthetic coloured ice cream

4.3.2.4. Changes due to pH

The pH of the fresh ice cream and yoghurt after 8 weeks of storage were observed and are given in the Table 16 & Fig.17. It showed that no significant change in pH for both natural coloured and synthetic coloured ice cream as well as yoghurt on storage was noted. Thus the changes in colour occurred on storage may not be due to the change in pH of the samples.

4.3.2.5. Comparison of marigold lutein with synthetic colour application

Ice cream

The mean rank of scores and percentage significance of sensory evaluation of synthetic and natural coloured freshly prepared ice cream samples are presented in Table 17.

The relative significance of the natural marigold colour with synthetic colours like lemon yellow and orange red in ice cream was observed by comparing the initial samples prepared for the most prominent parameters of ice cream namely colour, flavour, taste and consistency using the Kendall's coefficient of concordance test. The result revealed that the panel had unique opinion on the superiority of marigold colour in relation to lemon yellow and orange red colour in the fresh ice cream (Plate 12).

The products stored at -18°C at weekly intervals were compared with freshly prepared samples. For every week fresh and stored samples were compared separately for the three colours using Mann-Whity test. The mean rank of scores for the fresh and stored and also the percentage significance presented in Table 19 to 22. The percentage significance level was fixed at 20 percent for a broad based inference.

Initially marigold colour did not differ for the fresh and stored samples among the three colours. Subsequently there was an increased degradation of marigold colour over eight weeks of storage in comparison to the fresh sample starting from the 4th week onwards. In contrast lemon yellow did not show any degradation in colour in relation to fresh even after eight weeks of storage (Plate 13). Observations in orange red were in parallel to marigold colour (Table 19 & Fig. 18).

Flavour is one of the most attractive features of ice cream. The marigold preparation maintained its flavour afresh up to third week. But an immediate loss of flavour was observed subsequently. Observations in lemon yellow and orange red

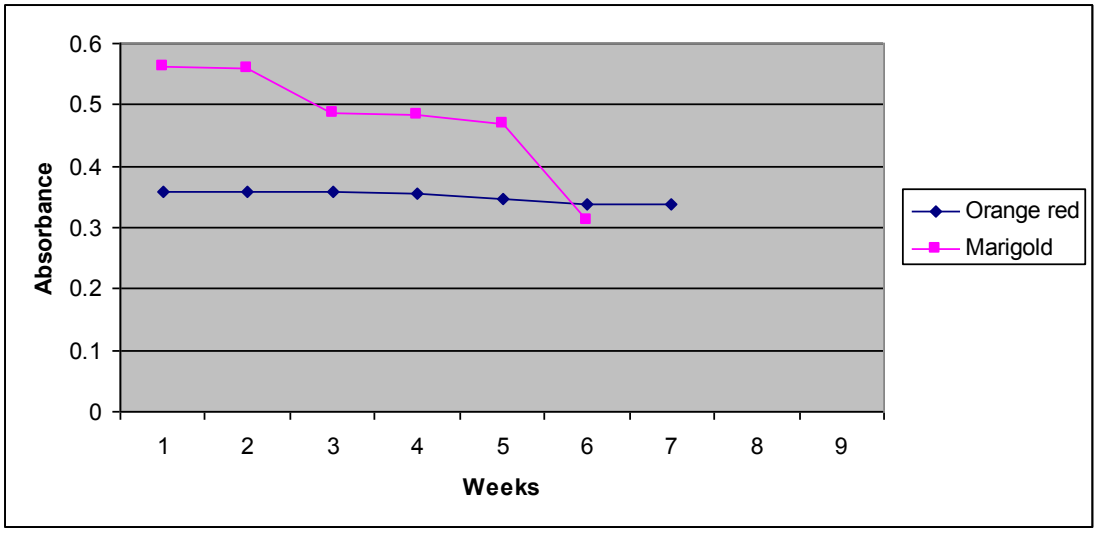


Fig.14. Effect of storage on colour of natural and synthetic coloured yoghurt

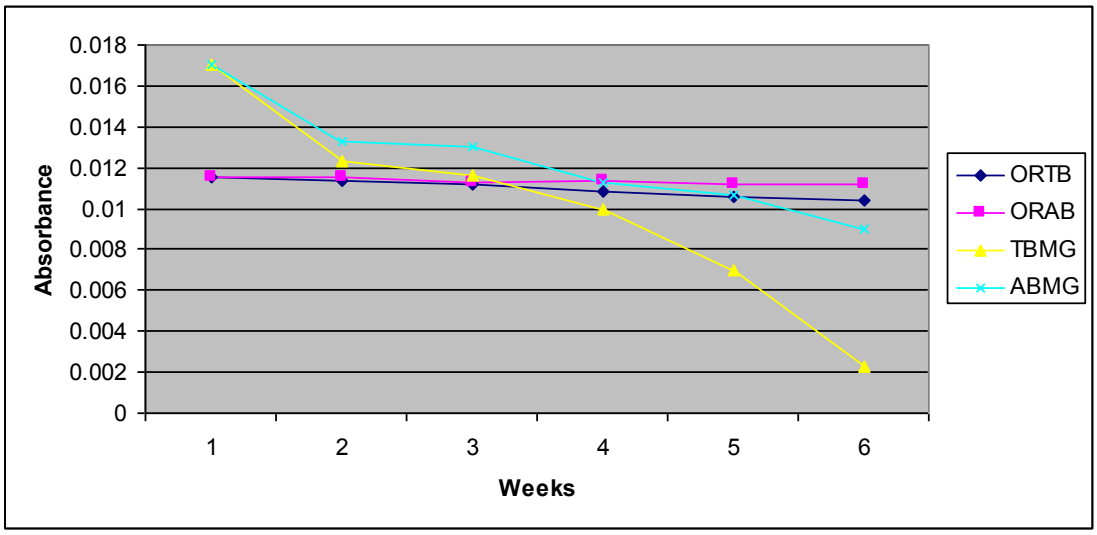


Fig. 15. Effect of light on colour of coloured oil during storage

ORTB- Orange red transparent bottle, ORAB-Orange red amber bottle, TBMG-Marigold colour transparent bottle, ABMG-Marigold colour amber bottle.

a)Changes due to light in natural and synthetic coloured oil

Marigold colour before storage

Marigold colour after storage

Orange red before storage

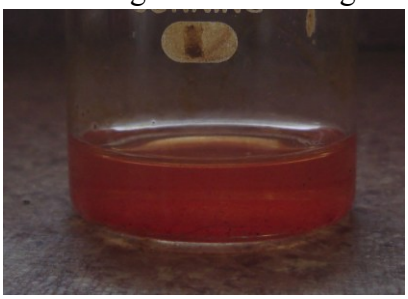
Orange red after storage

b)Changes due to heat in natural and synthetic coloured oil

Marigold before heating

Marigold after heating

Orange red before heating



Orange red after heating

Plate 11

Table .15. **Mean absorbance of synthetic and natural coloured oil on heating**

Time of heating (at boiling temperature) in minutes	**Absorbance at 446nm ***	
	Marigold colour	**Orange red**
0	0.017	0.0115
10	0.013	0.0114
20	0.008	0.0108
30	0.003	0.0104

*Mean value replication

Table 16. **pH of coloured fresh and stored ice cream and yoghurt**

Sample	**pH***			
	Ice cream		**Yoghurt**	
	Marigold colour	**Orange red**	**Marigold colour**	**Orange red**
Fresh	3.51	3.5	5.45	5.44
Stored	3.4	3.3	4.83	4.83

*Mean value replication

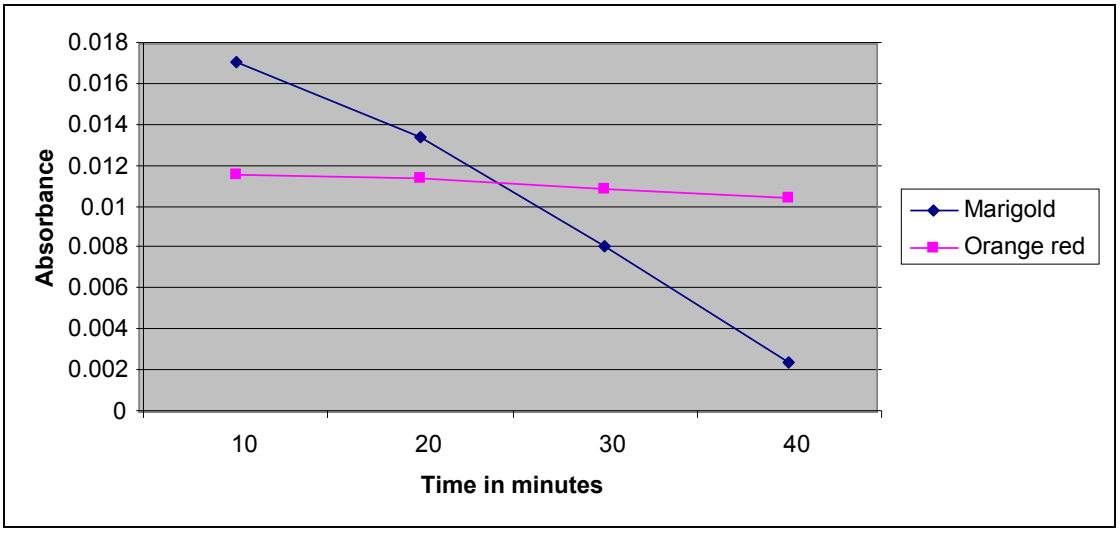


Fig .16. Effect of heat on colour of coloured oil

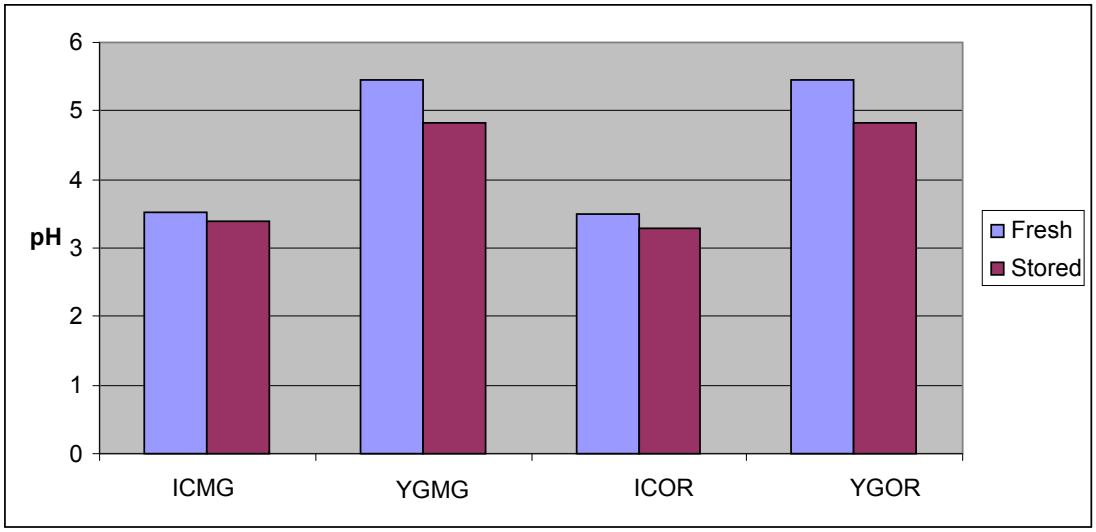


Fig. 17. Changes in pH during storages of marigold and synthetic coloured ice cream and yoghurt

ICMG- marigold coloured ice cream, YGMG- marigold coloured yoghurt,
ICOR - orange red coloured ice cream, YGOR- orange red coloured yoghurt

Table.17 . **Score for sensory attributes of natural and synthetic coloured fresh ice cream.**

Colour	Mean rank			
	Colour	Flavour	Taste	Consistency
LY	2	2	1.9	1.9
MG	2.9	2.4	2.5	1.9
OR	1.2	1.6	1.6	2.2
%Significance*	1.8	36.8	17.4	36.8

Table. 18. **Score for sensory attributes of natural and synthetic coloured fresh yoghurt**

Colour	Mean rank			
	Colour	Flavour	Taste	Consistency
LY	1.9	1.8	1.7	1.9
MG	2.9	2.9	2.9	2.2
OR	1.2	1.3	1.4	1.9
%Significance*	1.4	1.5	1.5	36.8

LY 0.01g lemon yellow
MG 0.2ml of lutein formulation
OR 0.01g orange red

Values are mean rank score based on Kendall's W Test.
*Significance level fixed at 20%

colours were almost in parallel to marigold colour. The superiority of natural colouration atleast for two weeks is much evident (Table 20).

Taste was maintained up to three weeks in the natural coloured sample while lemon yellow and orange red maintained only for a shorter period of two weeks (Table 20).

Consistency depends up on the mode of storage and serving. Orange red lost its consistency immediately after the first week where as lemon yellow and marigold were parallel in maintaining their consistency up to a higher duration (Table 22).

The weekly changes in sensory attributes observed in stored naturally and synthetic coloured ice cream is presented in Table 23.

Yoghurt

The Kendall's coefficient of concordance to observe the relative significance of the natural marigold colour with synthetic colours like lemon yellow and orange red in yoghurt revealed that the panel had the unique opinion on the superiority of marigold colour in relation to lemon yellow and orange red colour in yoghurt for all the parameters colour, flavour, taste and consistency (Table 18& Plate12).

The persistency and consistency of marigold colour over weeks of storage was assessed by an initial preparation and subsequent storage over six weeks. The synthetic colours namely lemon yellow orange red were also assessed in parallel to marigold colour.

For every week fresh and stored samples were compared separately for the three colours using Mann Whity test. The mean rank of scores for the fresh and stored as also the percentage significance presented in Table 24 to 27. The percentage significance level was fixed at 20 for a broad based inference.

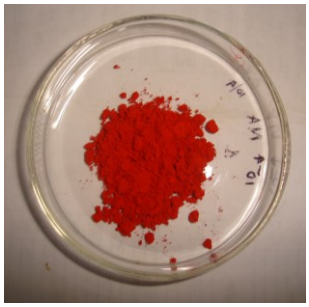
Initially marigold colour did not differ for the fresh and stored samples among three colours. Subsequently there was an increased degradation of marigold colour over six weeks of storage in comparison to the fresh sample from the second week onwards. In contrast lemon yellow did not show any degradation in colour even after six weeks of storage (Plate 13). Orange red showed significant degradation after fourth week of storage (Table 24 & Fig.19).

The marigold coloured preparation maintained its flavour up to second week. But an immediate loss of flavour was observed subsequently. In contrast lemon

Comparison of marigold colour with synthetic colours

Lemon yellow

Marigold



Orange red

Coloured ice cream

Lemon yellow Marigold Orange red

Coloured yoghurt

Lemon yellow Marigold Orange red

Plate 12

Changes due to storage

Coloured ice cream

LM MG OR

Fresh

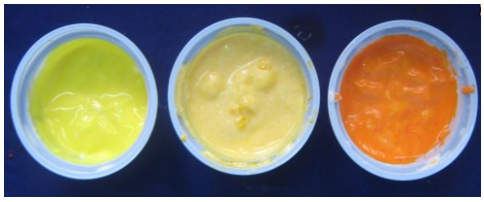
LM MG OR

After 8 weeks of storage

Coloured yoghurt

LM MG OR

Fresh



LM MG OR

After 6 weeks of storage

LM – lemon yellow, MG- marigold, OR- orange red

Plate 13

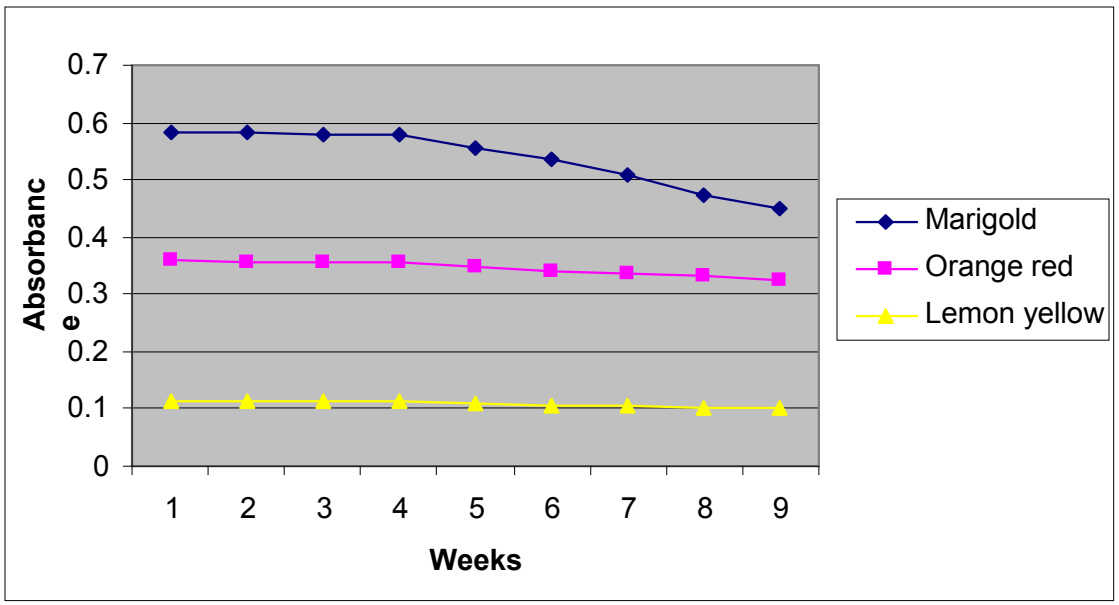


Fig. 18. Comparison of changes in colour during storage of natural vs. synthetic coloured ice cream

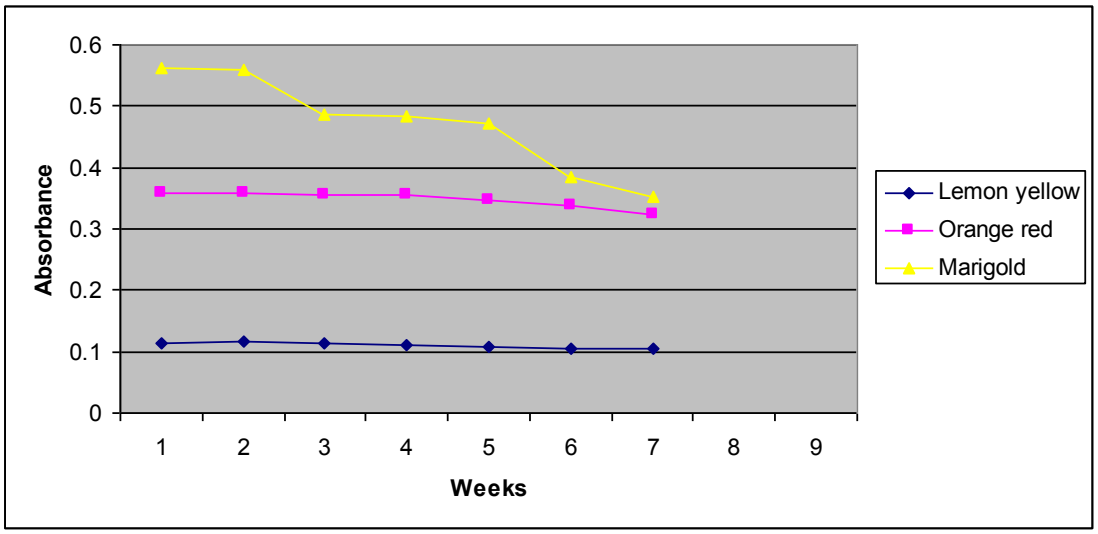


Fig. 19. Comparison of changes in colour during storage of natural vs. synthetic coloured yoghurt

yellow and orange red colours maintained up to fourth week. Subsequently slight decrease was found in the flavour (Table 25).

Taste was maintained by marigold coloured sample up to three weeks. Lemon yellow and orange red did not show any decrease in taste during the entire period of storage (Table 26).

Orange red lost its consistency immediately after the second week where as lemon yellow and marigold were parallel in maintaining their consistency up to fourth week (Table 27).

The weekly changes in sensory attributes observed in stored naturally and synthetic coloured yoghurt is presented in Table 28.

In brief an enhanced stability for the parameters of natural colouration atleast for a shorter period compared to synthetic colour was clear but maintenance of the stability in the natural colour against light, heat, etc. needs further studies.

Table 19. Sensory evaluation score for colour of natural vs. synthetic coloured ice cream

Storage period in weeks →		week1	week2	week3	week4	week5	week6	week7	week8
LY	Fresh	6.0	7.0	5.5	6.0	6.0	5.5	6.0	6.0
	Stored	5.0	4.0	5.5	5.0	5.0	5.5	5.0	5.0
	%Significance *	51.3	7.2	100	54.9	51.3	100	54.9	51.3
MG	Fresh	5.5	6.0	6.0	6.4	7.10	7.4	7.6	7.6
	Stored	5.5	5.0	5.0	4.6	3.9	3.6	3.4	3.4
	%Significance *	100	31.7	31.7	18	5.8	3.1	1.5	2.0
OR	Fresh	6.5	6.8	6.4	6.8	7.2	6.8	6.8	5.9
	Stored	4.5	4.2	4.6	4.2	3.8	4.2	4.2	5.1
	%Significance *	22.1	9.3	18	9.3	4.2	9.3	9.3	60.6

Values are mean rank score based on Mann-Whitney test for consistency
*** Significance level fixed at 20%**
% Significance less than 20 shows significant difference exist.

LY – Lemon yellow, MG – Marigold, OR – Orange red

Table 20. Sensory evaluation score for flavour of natural vs. synthetic coloured ice cream

Storage period in weeks →		**week1**	**week2**	**week3**	**week4**	**week5**	**week6**	**week7**	**week8**
LY	Fresh	5.5	5.0	6.5	6.5	6.5	6.7	5.5	6.2
	Stored	5.5	6.0	4.5	4.5	4.5	4.3	5.5	4.8
	%Significance *	100	54.9	13.4	22.1	13.4	16.6	100	41.9
MG	Fresh	5.5	5.5	6.0	6.4	6.5	7.0	7.7	7.6
	Stored	5.5	5.5	5.0	4.6	4.5	4.0	3.4	3.4
	%Significance *	100	100	51.3	18	22.1	7.2	1.4	1.5
OR	Fresh	5.9	5.5	6.5	6.8	7.2	6.8	5.5	7.1
	Stored	5.1	5.5	4.5	4.2	3.8	4.2	5.5	3.9
	%Significance *	22.1	100	22.1	9.3	4.2	9.3	100	5.8

Values are mean rank score based on Mann-Whitney test for consistency
* Significance level fixed at 20%
% Significance less than 20 shows significant difference exists.

LY – Lemon yellow, MG – Marigold, OR – Orange red

Table 21. Sensory evaluation score for taste of natural vs. synthetic coloured ice cream

Storage period in weeks →		**week1**	**week2**	**week3**	**week4**	**week5**	**week6**	**week7**	**week8**
LY	Fresh	5.0	5.5	6.5	5.1	6.1	5.9	6.6	5.9
	Stored	6.0	5.5	4.5	5.9	4.9	5.1	4.4	5.1
	%Significance *	54.9	100	13.4	60.6	50.2	60.6	18.9	63.5
MG	Fresh	6.0	6.3	6.0	6.4	7.2	7.6	7.4	7.2
	Stored	5.0	4.7	5.0	4.6	3.8	3.4	3.6	3.8
	%Significance *	51.3	33.9	51.3	18	4.2	1.5	3.1	5.4
OR	Fresh	5.5	6.0	6.5	6.0	6.4	5.5	6.4	6.8
	Stored	5.5	5.0	4.5	5.0	4.6	5.5	4.6	4.2
	%Significance *	100	51.3	13.4	31.7	18	100	4.21	22.2

Values are mean rank score based on Mann-Whitney test for consistency
* Significance level fixed at 20%
% Significance less than 20 shows significant difference exist.

LY – Lemon yellow, MG – Marigold, OR – Orange red

Table 22. Sensory evaluation score for consistency of natural vs. synthetic coloured ice cream

Storage period in weeks →		**week1**	**week2**	**week3**	**week4**	**week5**	**week6**	**week7**	**week8**
LY	Fresh	6.0	6.0	5.5	6.5	5.0	6.5	5.5	7.1
	Stored	5.0	5.0	5.5	4.5	6.0	4.5	5.5	3.9
	%Significance *	31.7	51.3	100	22.1	31.7	13.4	100	6.5
MG	Fresh	5.0	6.4	6.0	6.0	6.4	5.5	5.5	6.4
	Stored	6.0	4.6	5.0	5.0	4.6	5.5	5.5	4.6
	%Significance *	31.7	18	31.7	31.7	18	100	100	18.0
OR	Fresh	5.5	6.7	6.5	6.0	5.5	6.5	7.0	7.5
	Stored	5.5	4.3	4.5	5.0	5.5	4.5	4.0	3.5
	%Significance *	100	16.6	13.4	51.3	100	22.1	5.0	1.7

Values are mean rank score based on Mann-Whitney test for consistency
* Significance level fixed at 20%
% Significance less than 20 shows significant difference exist.

LY – Lemon yellow, MG – Marigold, OR – Orange red

Table 23. Weekly score for sensory attributes of stored natural and synthetic coloured ice cream

Storage period in weeks ↓	Colour			Flavour			Texture			Consistency		
	LY	MG	OR	LY	MG	OR	LY	MG	OR	LY	MG	OR
Week1	5.0	5.5	4.5	5.5	5.5	5.1	6.0	5.0	5.5	5.0	6.0	5.5
Week2	4.0	5.0	4.2	6.0	5.5	5.5	5.5	4.7	5.0	5.0	4.6	4.3
Week3	5.5	5.0	4.6	4.5	5.0	4.5	4.5	5.0	4.5	5.5	5.0	4.5
Week4	5.0	4.6	4.2	4.5	4.6	4.2	5.9	4.6	5.0	4.5	5.0	5.0
Week5	5.0	3.9	3.8	4.5	4.5	3.8	4.9	3.8	4.6	6.0	4.6	5.5
Week6	5.5	3.6	4.2	4.3	4.0	4.2	5.1	3.4	5.5	4.5	5.5	4.5
Week7	5.0	3.4	4.2	5.5	3.4	5.5	4.4	3.6	4.6	5.5	5.5	4.0
Week8	5.0	3.4	5.1	4.8	3.4	3.9	5.1	3.8	4.2	3.9	4.6	3.5

Values are mean rank score based on Mann-Whitney test for consistency
LY – Lemon yellow, MG – Marigold, OR – Orange red

Table 24. Sensory evaluation score for colour of natural vs. synthetic coloured yoghurt

Storage period in weeks →		**week1**	**week2**	**week3**	**week4**	**week5**	**week6**
LY	Fresh	6.0	5.50	6.0	6.5	6.0	6.0
	Stored	5.0	5.50	5.0	4.5	5.0	5.0
	%Significance *	54.9	100	51.3	22.1	54.9	54.9
MG	Fresh	6.0	6.0	6.8	7.7	6.9	7.7
	Stored	5.0	5.0	4.2	3.3	4.9	3.3
	%Significance *	51.3	51.3	9.3	1.4	11.8	1.4
OR	Fresh	5.9	6.3	5.9	6.8	6.3	7.1
	Stored	5.1	4.7	5.1	4.2	4.7	3.9
	%Significance *	60.6	33.9	65	9.3	33.9	5.8

Values are mean rank score based on Mann-Whitney test for consistency
* Significance level fixed at 20%
% Significance less than 20 shows significant difference exist.

LY – Lemon yellow, MG – Marigold, OR – Orange red

Table 25. Sensory evaluation score for flavour of natural vs. synthetic coloured yoghurt

Storage period in weeks →		**week1**	**week2**	**week3**	**week4**	**week5**	**week6**
LY	Fresh	5.5	5.5	6.0	6.0	6.0	6.0
	Stored	5.5	5.5	5.0	5.0	5.0	5.0
	%Significance *	100	100	31.7	51.3	51.3	51.3
MG	Fresh	6.0	5.5	6.8	6.8	6.8	6.8
	Stored	5.0	5.5	4.2	4.2	4.2	4.2
	%Significance *	31.7	100	9.3	9.3	9.3	9.3
OR	Fresh	6.0	6.0	6.3	6.0	6.0	6.0
	Stored	5.0	5.0	4.7	5.0	5.0	5.0
	%Significance *	51.3	51.3	33.9	51.3	51.3	51.3

Values are mean rank score based on Mann-Whitney test for consistency
* Significance level fixed at 20%
% Significance less than 20 shows significant difference exist.

LY – Lemon yellow, MG – Marigold, OR – Orange red

Table 26. Sensory evaluation score for taste of natural vs. synthetic coloured yoghurt

Storage period in weeks →		**week1**	**week2**	**week3**	**week4**	**week5**	**week6**
LY	Fresh	5.5	5.5	5.5	6.5	6.0	6.0
	Stored	5.5	5.5	5.5	4.5	5.0	5.0
	%Significance *	100	100	100	22.1	54.9	54.9
MG	Fresh	6.0	5.5	6.8	7.1	7.4	7.4
	Stored	5.0	5.5	4.2	3.9	3.6	3.6
	%Significance *	31.7	100	9.3	5.8	3.1	3.3
OR	Fresh	5.5	6.0	6.0	6.0	6.0	6.8
	Stored	5.5	5.0	5.0	5.0	5.0	4.2
	%Significance *	100	51.3	51.3	31.7	31.7	51.3

Values are mean rank score based on Mann-Whitney test for consistency
* Significance level fixed at 20%
% Significance less than 20 shows significant difference exist.

LY – Lemon yellow, MG – Marigold, OR – Orange red

Table 27. Sensory evaluation score for consistency of natural vs. synthetic coloured yoghurt

Storage period in weeks →		**week1**	**week2**	**week3**	**week4**	**week5**	**week6**
LY	Fresh	6.0	5.5	6.0	6.0	6.5	7.5
	Stored	5.0	5.5	5.0	5.0	4.5	3.5
	%Significance *	31.7	100	31.7	31.7	13.4	1.7
MG	Fresh	6.0	3.0	6.0	6.0	6.4	7.2
	Stored	5.0	5.0	5.0	5.0	4.6	3.8
	%Significance *	31.7	31.7	31.7	31.7	18	4.2
OR	Fresh	5.5	5.5	6.5	7.5	7.1	8.0
	Stored	5.5	5.5	4.5	3.5	3.9	3.0
	%Significance *	100	100	13.4	1.4	6.5	0.5

Values are mean rank score based on Mann-Whitney test for consistency
* Significance level fixed at 20%
%Significance less than 20 shows significant difference exist.

LY – Lemon yellow, MG – Marigold, OR – Orange red

Table 28. Weekly score for sensory attributes of stored natural and synthetic coloured yoghurt

Storage period in weeks ↓	Colour			Flavour			Texture			Consistency		
	LY	MG	OR	LY	MG	OR	LY	MG	OR	LY	MG	OR
Week1	5.0	5.0	5.1	5.5	5.0	5.0	5.5	5.0	5.5	5.0	5.0	5.5
Week2	5.5	5.0	4.7	5.5	5.5	5.0	5.5	5.5	5.0	5.5	5.0	5.5
Week3	5.0	4.2	5.1	5.0	4.2	4.7	5.5	4.2	5.0	5.0	5.0	4.5
Week4	4.5	3.3	4.2	5.0	4.2	5.0	4.5	3.9	5.0	5.0	5.0	3.5
Week5	5.0	4.9	4.7	5.0	4.2	5.0	5.0	3.6	5.0	4.5	4.6	3.9
Week6	5.0	3.3	3.9	5.0	4.2	5.0	5.0	3.6	4.2	3.5	3.8	3.0

Values are mean rank score based on Mann-Whitney test for consistency

LY – Lemon yellow, MG – Marigold, OR – Orange red

Discussion

Discussion

Natural colours are gaining importance these days owing to the growing public demand for natural things against synthetic chemicals. Consumer pressure, sociological changes and technological advances in the food processing industry have increased the demand for natural colours (Choudhuri *et al*., 2004).

Though synthetic dyes possess intense colouring strength, good solubility and high stability easiness in processing, its harmful effects make people to limit the consumption. The safety in natural colour and its health benefits including prevention of cancer, age related macular degeneration, protection against oxidant activity etc. make it preferred by the consumers (Zhang *et al*., 1991; Seddon *et al*., 1994; Chew, 1996).

Experiments on colour extraction, preservation and its application on food were done with this background to develop appropriate technology for the production of natural colour from marigold flower. The results are discussed hereunder.

5.1. Extraction of colour from marigold

5.1.1. Preparation of raw material

Petals of the flowers were separated and used as the substrate for the extraction, though whole flowers can be utilised for the purpose. Our experiment was restricted in the use of petals alone as the purpose of the colour extracted was food application. Navarrete-Bolanos, (2004) stated that when whole flowers were used, the solid residue of the extract was high and the colour obtained was used only for poultry feed.

Average yield of petals from the whole flowers was 62.5 percent. One batch of petals was subsequently subjected for curing, while the other tried with out curing for extraction purpose.

5.1.1.1. Curing

In the extraction methods tried, cured flowers yielded more lutein with less impurity than uncured flowers (Fig. 19, 20).

Navarrete-Bolanos, (2004) has reported that the efficiency of extraction depends mostly on the appropriate pre-treatments given to the flowers. Curing increases cell wall permeability and facilitate diffusion mechanism of mass exchange.

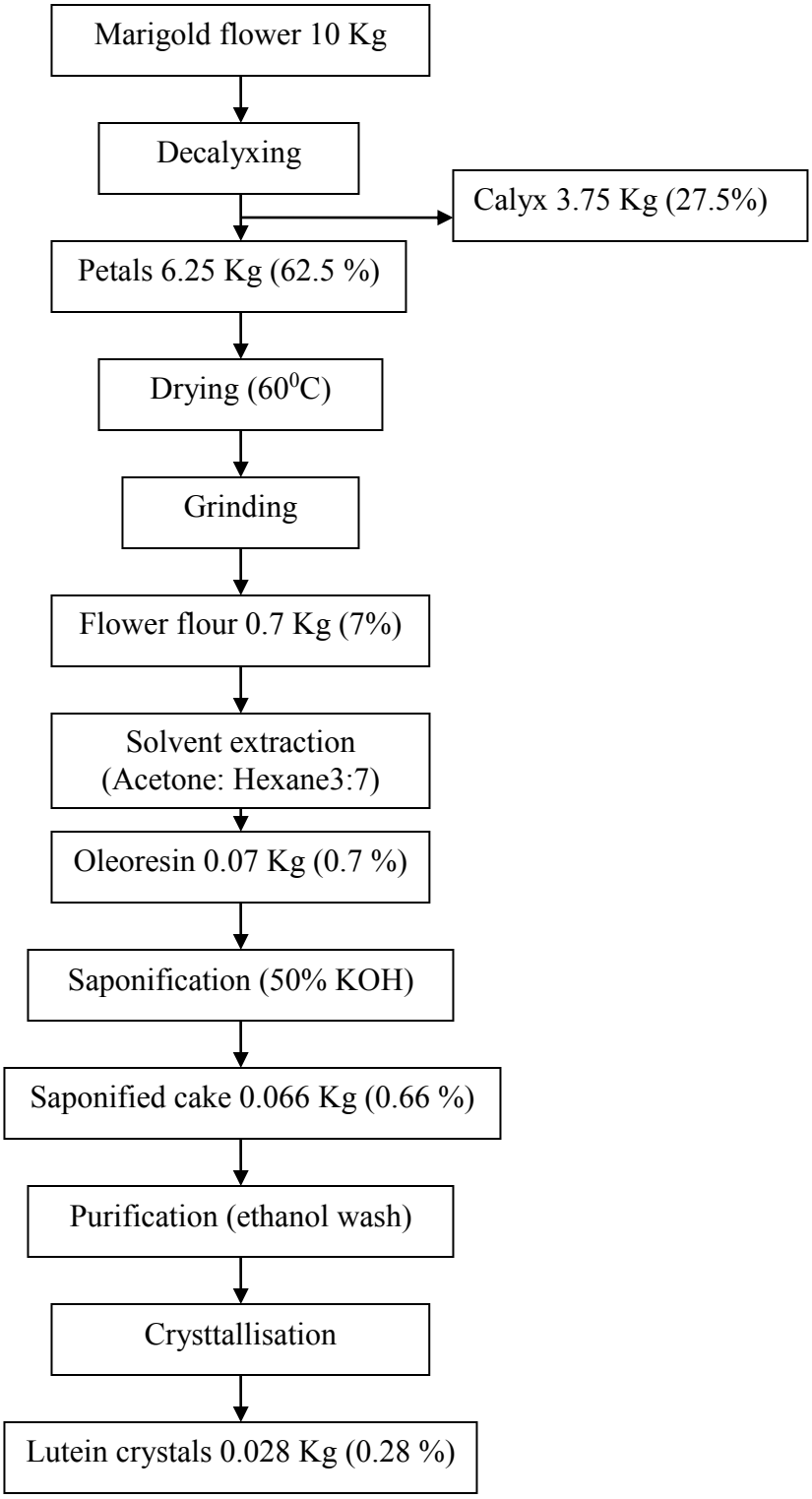


Fig.19. **Percentage recovery of lutein from uncured sample**

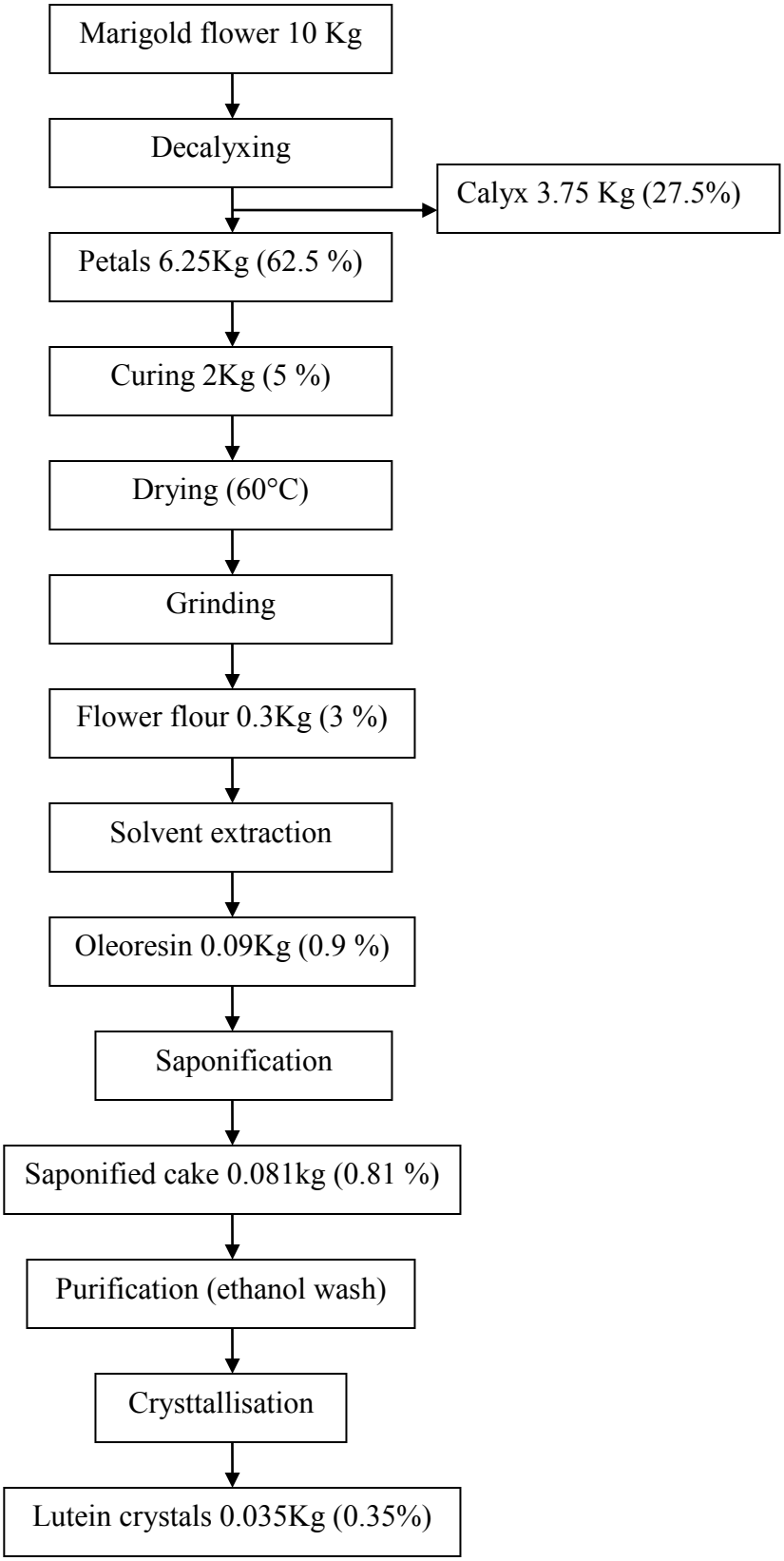


Fig.20. **Percentage recovery of lutein from cured sample**

Navarrete-Bolanos, (2004) has also reported that the ensilage (curing) is critical in the efficiency of the all over process of the colour extraction from marigold.

Micro organisms associated with the marigold flowers during ensilage are most significant which exhibits high cellulase activity. This resulted in an increased yield of the total xanthophyll extracted from the cured flowers than uncured flowers.

Similar results were reported by Delgado-Vargas and Paredes-Lopez, (1997) that the enzymatic treatment using commercial cellulase increased the yield in the colour. Probably this might have been the reason for getting three times more yield in colour from cured flowers than that of uncured flowers. Similar results were also reported by Navarrete-Bolanos, (2004).

5.1.2. Extraction of colour

Four methods of extraction were tried *viz* mechanical extraction, hot water extraction, acidified aqueous extraction and solvent extraction. The results are discussed here.

5.1.2.1. Mechanical extraction

Mechanical extraction of flowers yielded negligible amount of lutein and high amount of impurity. The content of lutein in the extract was only 1.03% from uncured and 1.53% from cured flowers.

Krishnamurthy, (1993) extracted annatto dye from *Bixa orellena* L., by macerating the seeds and staining the product through a sieve and drying. But complete extraction of pigment from marigold was not possible by this method.

5.1.2.2. Hot water extraction

Lutein, the pigment present in marigold petals is a fat soluble one. Hot water extraction yielded very low amount of colour and contained high amount of impurity. The content of lutein in the extract was only 1.86% from uncured and 3.33% from cured flowers

Tsimidou and Tsatsaroni, (1993)extracted crocin the carotenoid pigment from saffron using water, Bechtold *et al.,*(2006) extracted natural dye from berries and vegetables with boiling water and Diouf *et al*.,(2009) reported the extraction of *Pica mariana* by reflexing with water. But these pigments were water soluble in nature could be extracted using hot water. Lutein is a fat soluble pigment and hence it was concluded that lutein could not be extracted successfully using hot water.

5.1.2.3. Acidified aqueous extraction

In order to modify the hot water extraction acidified aqueous extraction was tried using 4% citric acid and the recovery of colour was 18.5% and 5.4% from cured and uncured petals respectively. Thus the result was higher compared to that of hot water extraction. This increased yield of colour may be due to the addition of citric acid which might have enhanced the permeability of lutein from the petals. Similar results were reported by Debicks- Perpisal *et al.,* (1983) in Annatto. Anu, (2009) in malay apple when 4% of citric acid was used for the extraction, better yield was obtained.

5.1.2.4. Solvent extraction

On comparison with the above methods solvent extraction using organic solvents yielded maximum lutein (Fig. 22).

Similar results were reported by Gierhart, (1994). According to Gierhart maximum marigold pigment can be extracted with suitable solvents. The results of our experiments were in agreement with these findings of Gierhart (1994).

Solvent used in the present extraction was hexane with acetone in 7:3 ratio. Solvent extraction yield of oleoresin was 10.12% from uncured sample and 30.2% from cured sample and the lutein content in the extracts were 22.86% and 64.26% in uncured and cured samples respectively. This higher yield of lutein could be due to the reason that lutein is a fat soluble pigment.

The ratio of solvents hexane: acetone as 7:3 was as suggested by Ranganna, (1986). This ratio of the solvent increased the solubility of pigments in the solvent.

The extraction was carried out as batches in a soxhlet apparatus and thus the quantity of solvents used was much reduced. Similar results were reported by Piccaglia *et al.,* (1998) and Navarrete-Bolanos, (2001) in marigold.

5.1.3. Purification

The marigold flowers are always in an esterified form with fatty acid and not in a free form. There for purification is an essential step to obtain pure crystalline lutein from the oleoresin obtained through solvent extraction.

Pure crystalline lutein can alone be used for human consumption as suggested by ausich and sanders, (1997)., Khachick, (2001)., Levi, (2001)., and Madhavi and Kagan,(2002).

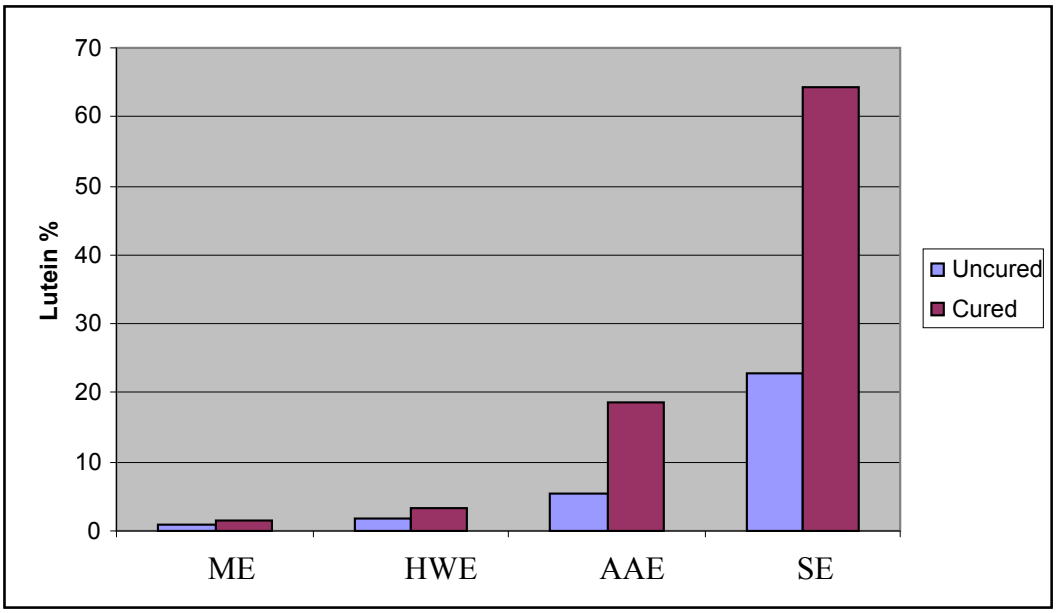


Fig. 22. Lutein recovery from different extraction methods of cured and uncured samples

HWE- Hot water extraction, AAE- Acidified aqueous extraction, SE- Solvent extraction

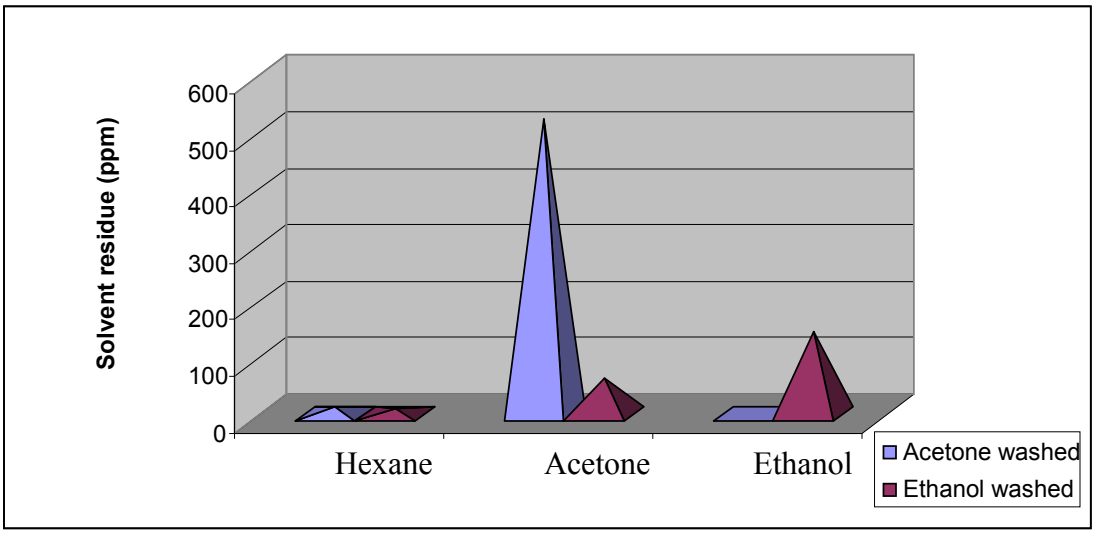


Fig. 23. Residual solvent content of purified lutein products

5.1.3.1. Saponification

Saponification was a well established method to convert the fatty acid esters of lutein to free lutein. Here the fatty acid esters of lutein in oleoresin was converted to free lutein removing chlorophyll, unwanted waxes, lipids and other interfering substances from lutein.

The cake obtained after saponification was further washed with either acetone or ethanol and dried to get pure crystalline lutein. The yield of pure lutein obtained through washing with ethanol or acetone was on par in both with 38% yield. Similar results were obtained by Rosales and Cardona, (2006).

5.1.3.2. Purity test

According to the recommendations suggested by Cantrill,(2004); Joint Expert Committee on Food Additives (JECFA), (2004) and Khachik, (2009) those samples containing pure lutein of 90% and above alone could be used in food applications. The purity of lutein obtained in the present experiment was much higher to the specification suggested above. Hence it can very well be used in the food applications.

5.1.3.3. Solvent residue test

The residual solvent remaining in the extract was estimated using GCMS method and found that the residual acetone was much above the maximum limit prescribed by JECFA. Where as that of ethanol is in the safe level and hence ethanol washed lutein alone was used for further food applications (Fig. 23).

5.2. Preservation of colour

Some of the natural colour preparations are available in traditional liquid forms which are not stable during storage. To overcome this problem preparations like solution, suspension, powder and tablets in high concentrations are made available. They have the advantages like improved stability, uniform mixing, and added stability during storage (Apaenathi and Borkhatriya, 1999).

Taylor, (1984) prepared natural colours in powder form by their attachment to carrier substances. Carriers were selected depending on the nature of the colour and the product in which the colour has to be applied.

In the present study lutein extracted from marigold was preserved as an solution using soyabean oil.

Phillip and Berry, (1975) has reported that lutein is soluble in vegetable oil to an extent of 20% at 60ºC and completely miscible with vegetable oil above 80ºC.

They have prepared concentrated solutions of carotenoids in essential oils and cosmetic oils for use in food, beverages, and personal care products.

Commercial lutein production in vegetable oil like soyabean oil has already been well approved.

Oxidation stability, antioxidant effectiveness and effective preservation of pigments of soyabean oil are reported by Yen and Chen, (1995); Gouveia *et al*., (2007). There for soya bean oil was found to be an excellent vegetable oil to preserve the lutein and its easiness in application also facilitated further use in the present study (Fig. 24). Among the different concentrations of lutein oil solution 1:2 (lutein: oil) sample with 500 ppm lutein was found to be best for the use in food because of its high colouring property, good consistency, and low oil content. This concentration is much higher than the minimum 20% concentration suggested by Food Standards Australia New Zealand (FSANZ), (2009) in the specifications for lutein: oil suspension.

5.3. Application and storage studies

5.3.1. Safety for application in food

Increasing health awareness and corresponding lifestyle influence the development of new food products especially carotenoid formulations with processing characteristic and stability to meet the customer's requirements (Sambale., 1999)

Reports by Ausich and Sanders, (1997)., Khachik, (2001)., Levi, (2001) and Madhavi and Kangan, (2002), Kruger *et al.*,2002 suggested pure crystalline lutein for human consumption either as nutritional supplement or as food additive.

According to USDA and US DHHS, (2000), carotenoid from marigold (*Tagetes erecta*) was possible to be added in food and was generally recognised as safe (GRAS)

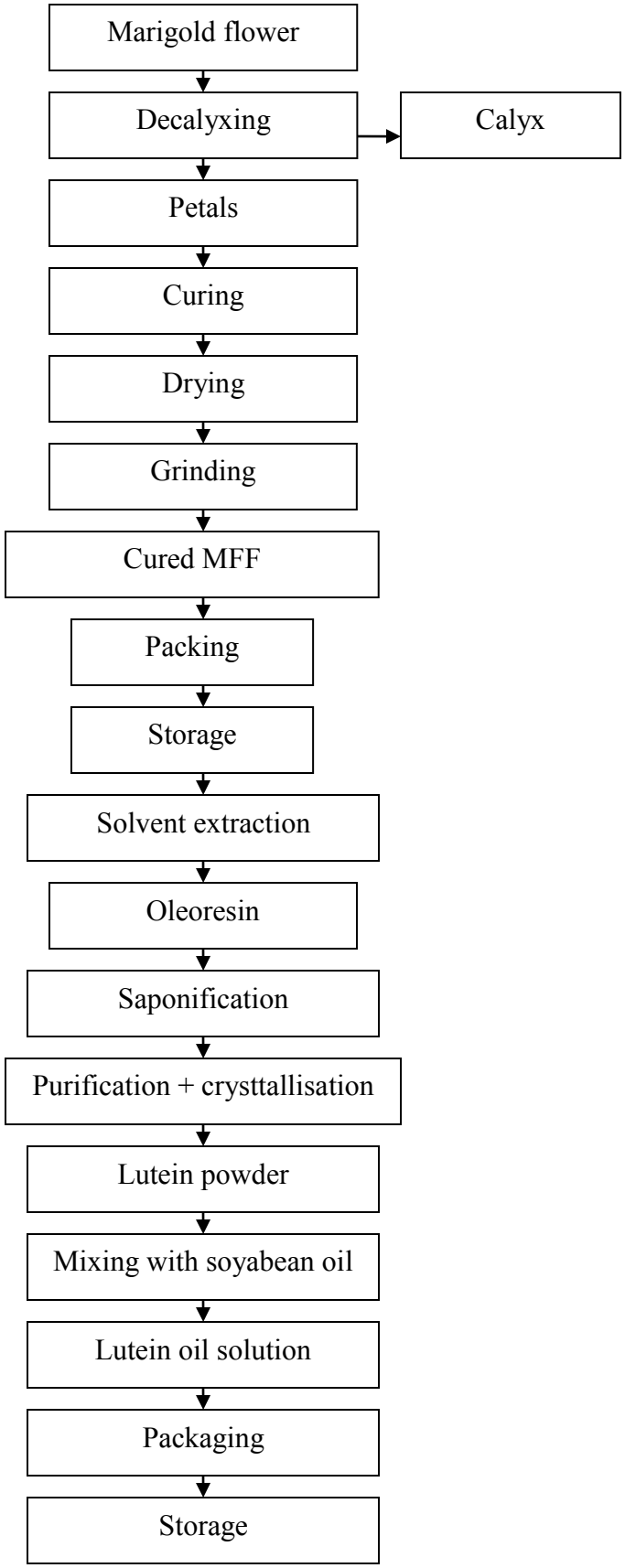


Fig.24 **Steps in isolation, purification and development of lutein formulation from marigold.**

5.3.2. Application in food

Lutein solution was applied in food products viz ice cream, milk, yoghurt, oil and juice. The results showed that the lutein solution is found to be appropriate to be used in ice cream yoghurt and oil as it contains less amount of water and high amount of fat. While it was not suitable to use in milk and juice because of its fat soluble nature .Where ever fat is less and water is more , it will not properly mixed up to give a uniform colour to the product.

Proper formulations added to get uniform colouration for the product revealed that concentration of 0.1g of lutein was found to be the best level for use in ice cream and yoghurt where as slightly higher concentration of 0.15g appears to be better for the use in oil. The concentrations of formulations in these products were similar to the concentrations reported by Cantrill, (2004).

Dietary intake data reported by JECFA, (2004) from a number of studies in North America and UK also agrees with the finding of the present experiment.

5.3.3. Storage study

Stability of lutein in the food products during storage is important to make the final recommendations. Degradation of lutein not only affects colour, it may also affect the flavour, texture, consistency etc. The results of various changes during storage due to light, temperature, pH with respect to stability of natural coloured products in comparison with synthetic colour are discussed here.

5.3.3.1. Changes due to storage time

In the case of ice cream degradation of natural colour was found from the forth week onwards were as in yogurt it started from second week itself. From these results it can be observed that colour of lutein solution will be stable only for a period of one month. After that the colour will gradually get faded.

Similar results were reported by Chantropornchai *et al.* (1999) in coloured mayonnaise.

Johns *et al.* (2005) reported that the cheddar cheese coloured with lutein showed a significant reduction in the colour after 12 weeks of storage compared to the storage with respect to the marginal colour value.

According to the report by Aryana *et al.,* (2006) in the interaction effect between lutein content and storage time is significant.

In the present case the colour of products were stable up to one month however it did not affect the taste, consistency and flavour of the product.

Similar results were obtained by Aryana *et al.* (2006) in the storage study of coloured strawberry yoghurt.

The synthetic coloured samples did not show any significant change during the storage, indicating its stability compared to natural colour. Similar results were reported by Sowbhagya *et al*. (2005) in a comparative study of turmeric colour and tartrazine.

5.3.3.2. Changes due to light

In order to study the effect of light on the stability of colour, natural and synthetic coloured oil samples were stored under transparent and amber coloured bottles and kept exposed to light. Natural coloured samples stored in transparent bottles faded faster than that of amber coloured bottles.

Tendency towards the deterioration of natural colour in food products on exposure to the light during storage was reported by several scientists (Klaui and Bauernfeind, (1981); Peseck and Worthsen, (1987); Delgado-Vargas *et al.* (2000); Sowbhagya *et al.* (2004); Aman *et al*. (2005).

It was also reported that carotenoid colour in mayonnaise absorbed light during its storage resulted in degradation of colour (Lennersten and Lingnert., 2000).

This might be the reason that the colour in the transparent bottle degraded very fast compared to amber bottle, which might have prevented the colour penetration in the product. Thus product has to be packed in opaque materials to retain the colour.

The samples with synthetic colour did not show significant changes in colour both in amber coloured and in transparent bottle indicating the stability of synthetic colour to light compared to natural colour. Similar results are reported by Cevallos-Casals and Cisneros-Zevallos, (2003).

5.3.3.3. Changes due to heat

Fast degradation of colour in coloured oil during heating shows the instability of lutein towards heat.

Khachatryan, (2003) have reported that heat has considerable effect on the stability of lutein.

The synthetic colour did not show significant reduction in colour during heating. Heat stability of synthetic colour compared to natural colour is reported by Cevallos-casals and Cisneras-Zevallos, (2003)

Thermal degradation of carotenoids like carotene, lutein etc have reported by Aman *et al*. (2005) and Perez-Galvez *et al.,* (2005) also have the same result in paprica oleoresin. Thus high temperature processing is not recommended for lutein added products.

5.3.3.4. Changes due to pH

No significant change in pH of the stored ice cream was observed during the entire period of storage. There for any change in the natural colour during storage can not be attributed to the effect of pH

Salem *et al,* (2009) has studied the effect of pH on degradation of dyes and observed that no change was obtained in acidic medium and slow rate of change in neutral pH.

According to Mendi *et al*. (2000) natural food colours are stable with in pH range 3.53 to 6.85. Since these products falls with a pH of 3.4 to 5.4 which is fairly acidic to neutral, no significant changes can occur in short storage period of one month. Similar results were reported by Jones *et al.* (2005) in cheese stored for 24 weeks.

5.4. Comparison of natural colour with synthetic colour

Among synthetically coloured and marigold coloured ice cream and yoghurt samples, the marigold samples were identified as the best with most appealing colour, taste, flavour, and consistency by the trained panel through sensory evaluation.

According to FPO specifications, synthetic colour @ 0.2g/kg (200ppm) is the permissive limit. Same amount of each colour was added to all the samples and lemon yellow coloured sample was too light in colour where as the samples added with orange red was too intense in colour. The natural coloured samples appeared with moderate colour and was appealing with no off flavour or taste. Kendall's coefficient of concodence also revealed the superiority of marigold colour over synthetic colours.

Sensory analysis of ice cream during the storage period revealed that , though initially marigold colour did not differ for any sensory quality, there was some change subsequently from the fourth week onwards were as the synthetic colour (lemon yellow) did not show any significant change in colour. But orange red showed certain changes in the final stages of its storage.

Similar was the case with yoghurt where marigold colour maintained the sensory quality up to second week. Afterwards changes were observed from the third week onwards were as the synthetic colour (lemon yellow) did not show any

significant change in colour. But orange red showed certain changes in the final stages.

Similar results were reported by Soubhagya *et al*., (2005) when compared the curcumine and synthetic tartrazine applied on expanded extruded balls from corn and soybean. Curcumine showed a faster rate of degradation compared to tartrazine. Thus the present result is in agreement with those findings. So it can be concluded that natural colours showed faster degradation than synthetic colours when added in food products during storage. In case of flavour, taste and consistency marigold preparation maintained the quality up to third week in ice cream and up to second week in yoghurt. But the synthetic colours failed to maintain its quality after second week. So the superiority of natural coloured preparation atleast for a shorter period was evident.

There for future line of work needs to impart stability to the natural colours when applied in the food under different conditions of light, temperature, pH etc.

Summary

Summary

Present investigation on extraction, preservation and utilization of natural colour from marigold (*Tagetus erecta L.*) was undertaken at the department of processing technology, College of Horticulture Vellanikkara during the period 2007-2009.

The objectives were to develop appropriate technology for extraction and preservation of natural colour from marigold and to assess its application in food and further stability during storage.

Protocol for extraction of natural colour was developed. Solvent extraction of cured marigold flower flour using acetone: hexane (3:7) in a batch process yielded oleoresin with highest lutein and less impurity. Oleoresin was saponified using KOH. The cake obtained after saponification was further purified by ethanol wash to yield pure crystalline lutein powder.

The extracted colour from marigold was preserved as a solution in the ratio 1:2 lutein: soyabean oil mix was found to be best for the use in food because of its high colouring property, good consistency and low oil content. Solution was packed in glass bottle and stored at room temperature.

Lutein solution was applied in food products viz. ice cream, yoghurt, milk, oil, jam and juice. Products having more amount of fat, the colour got properly mixed giving a uniform colour. Thus it was suitable for ice cream, yoghurt and vegetable oil but doesn't suit in milk, jam and juice as it contained more amount of water.

The concentration of 0.1g of lutein was found to be the best level for use in ice cream and yoghurt where as 0.15g appeared to be better for the use in oil.

The study of various changes during storage due to light, temperature, pH with respect to stability of natural coloured products in comparison with synthetic colour revealed that the colour of the natural products will be stable only for a period of one month after that the colour will gradually get faded. The synthetic coloured samples did not show any significant change during the same period of storage.

Natural coloured oil stored in transparent bottles degraded very fast compared to those stored in amber coloured bottles. Thus the products have to be packed in

opaque materials to retain the colour. Synthetic coloured oil was stable to light compared to natural colour.

Natural coloured oil degraded on heating, so high temperature processing is not recommended for lutein added products where as synthetic colour did not show any significant reduction in colour on heating.

No significant change in pH of the product was observed during storage. Therefore any change in natural colour was not due to the change in pH.

Sensory analysis of natural coloured as well as synthetic coloured ice cream and yoghurt revealed that natural coloured products were superior in all the characters like colour, flavour, taste and consistency.

During storage natural colour showed faster degradation than synthetic colour. In case of flavour, taste, and consistency marigold coloured products maintained the quality up to third week in ice cream and second week in yoghurt. But synthetic colours fail to maintain its quality after second week. So superiority of natural coloured products atleast for a shorter period was evident. But an enhanced stability of colour under different conditions of light, temperature and pH are to be developed.

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Appendix

Name of the Scorer:

Product:

Please score the given products using the following 5 point Hedonic scale

Score	**Inference**
5	Like very much
4	Like
3	Neither like nor dislike
2	Dislike
1	Dislike very much

Product Code	Colour	Taste	Flavour	Consistency

Remarks: (Please write which flavour is dominating, weather you find the colour appealing.)

Signature:

Abstract

Extraction, preservation, and utilization of natural colour from marigold (*Tagetus erecta* L.)

By

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(2007-12-101)

ABSTRACT OF THE THESIS

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(PROCESSING TECHNOLOGY)

Faculty of Agriculture

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DEPARTMENT OF PROCESSING TECHNOLOGY

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Abstract

A study on extraction, preservation, and utilization of natural colour from marigold (*Tagetus erecta L.*) was conducted at the Department of Processing Technology, College of Horticulture, Vellanikkara during 2007-2009.

Method for extraction of natural colour from marigold was developed. Solvent extraction of cured marigold flower flour using acetone: hexane in the ratio 3:7 was found to be the best method. The extracted oleoresin was purified by saponifying with KOH and further ethanol wash to obtain pure crystalline lutein powder.

The extracted colour was preserved as 1:2 lutein: soyabean oil solution packed in glass bottles and stored at room temperature.

Lutein solution was found suitable for the application in the products like ice cream, yoghurt, oil etc. as these products contained high amount of fat where as found not suitable for aqueous products as this pigment is not water soluble. A concentration of 0.1 g gave the best colour in ice cream and yoghurt while 0.15g for oil.

Natural coloured products were stable only for a short period compared to synthetic colour during storage also it degrade faster on exposure to light, heat compared to synthetic colours. The changes in natural colour was not due to the changes in pH of product but due to the external agents like light and heat.

Sensory analysis of natural coloured as well as synthetic coloured ice cream and yoghurt revealed the superiority of natural colour in all the characters like colour, flavour, taste and consistency. During storage natural colour degraded faster than synthetic coloured products. Thus an enhanced stability of the colour with respect to light , heat etc. are to be developed to use these natural colour as a substitute for the synthetic colours.