CHAPTER 1

General Introduction

In the recent years, the production and application of synthetic dyes has released vast amount of waste and unfixed colourants that causes serious health hazards to human and disturbance to the eco-balance of nature (Jothi, 2008). The usage of synthetic dye such as tartrazine (food Yellow no. 4) has brought negative effects to human health such as skin rashes, hay fever, breathing problems, anxiety, migraines, depression, general weakness, heat waves and sleep disturbance (Hardwin, 2010). A petition was submitted to the Food and Drug Administration (FDA) in 2008, to ban the eight most widely used food colourants namely Yellow 5, Red 40, Blue 1, Blue 2, Green 3, Orange B, Red 3, and Yellow 6 (Consumers Affair, 2010) because these dyes are believed to cause hyperactivity and behavioural problem in children. The havoc in the usage of synthetic dyes has resulted in the urgency to resource for natural colourants as alternatives to the synthetic one.

Some of the commonly found natural colourants are annatto extract, beet powder, caramel, fruit juice, paprika, saffron, turmeric, and vegetable juice. However, several factors need to be taken into consideration for the potential source of natural colourant such as their abundance in nature, economic value and safety for consumption. Carotenoids from plants have been studied as potential sources for natural colourants ranging from yellow to red colour (Setiawan *et al.*, 2001). The significance and importance of carotenoids has led to various attempts by researches to study their compositions and functions from fruits (apple, grape, peach and plum), vegetables (lettuce, kale, corns and carrots), egg products and plants (Perry *et al.*, 2009; Kim *et al.*, 2007).

The *Ipomoea batatas* or commonly known as sweetpotato plant was described by Villareal (1982) as the world most under-rated crop and best kept secret in the agriculture field. *I. batatas* ranked seventh in the food crop production and one of the two major tuber crops in Asia, the Pacific and Ocenia region (Nissila *et al.,* 1999). The storage roots of the *I. batatas* plants are targetted as cheap and starchy staple in developing countries (especially during disaster relief) but the leaves of these plants are discarded during the harvesting season (Hall *et al*., 1998; Tanganik *et al*., 1999).

A previous study has demonstrated that the *I. batatas* leaves contained a comparable level of lutein (yellow colour pigments) with vegetables such as spinach and kale, both currently ranked highest in their lutein contents (Ishiguro and Yoshimoto, 2007). However, compared to both kale and spinach, the *I. batatas* leaves has a lower market value and hence, suitable as an alternative source of natural yellow dye. Currently approximately 95% – 98% of the *I. batatas* leaves are discarded after the roots are harvested. The lutein content in these leaves is however depends on the harvest date, cultivation conditions and cultivars (Ishiguro and Yoshimoto, 2007). To date, no study has been conducted to compare and determine the lutein and β-carotene levels in the different local varieties of *I. batatas* leaves in Malaysia.

Currently, the production of natural yellow dye available in the market is from African marigold (*Tagetes erecta*) which is a major source of carotenoids and lutein (Jothi, 2008; Prateesh *et al*., 2009). However, the main drawback of the African marigold is their seasonal nature, which deterred constant supply of these raw materials throughout the year to the natural dye industry. This is further coupled with the opening of new lands for cultivation and a high labour costs for maintenance, thus contributing to the

high cost of production. Hence, the exploitation of traditional crops grown with low inputs (especially in developing countries) would be a step towards better resource utilisation for growing consumer demands.

In this study, six different local varieties of *I. batatas* leaves will be studied namely *Ipomoea batatas var*. Batu Kelantan, *Ipomoea batatas var*. Batu Biasa, *Ipomoea batatas var*. Biru Putih, *Ipomoea batatas var.* Oren, *Ipomoea batatas var*. Vitato and *Ipomoea batatas var.* Indon. The storage roots of these varieties are commonly found in the market in Malaysia and are often consumed by Malaysians. Many studies have been focussing on the *I. batatas* storage roots but little is known about the leaves of this plant.

This study consists of four important parts: firstly, the morphological variations of the leaf and storage root of the different varieties of *I. batatas* will be studied followed by the general screening of the chlorophyll *a*, chlorophyll *b*, lutein and β-carotene contents in the leaves. The general screening will allow the identification of the variety with the highest level of lutein and β-carotene to be used for extraction. Besides that, the optimum storage temperatures (highest percentage of lutein and β-carotene pigments remained during storage) for the leaves prior to extraction will also be determined.

Previous attempts in carotenoids extraction have been performed using several chromatography methods such as high performance liquid chromatography, liquid chromatography-mass spectrometry, thin layer chromatography and column chromatography (Takaichi *et al*., 2010; Frenich *et al*., 2005). However, these methods are often expensive and require skilful technicians for operation (Küpper *et al*., 2007). Hence, in this study, the solvent-solvent extraction method is used to extract the yellow pigments from the *I. batatas* leaves. In the second part of this study, the optimum

extraction conditions for the leaves will be determined to maximise the yellow pigments content in the final extract. This is followed by the stability study on the pigments in the different storage conditions.

Qualitative analysis using liquid chromatography mass spectrometry / mass spectrometry method will be used to determine the composition of the extracted yellow pigments in the third part of this study. Besides, antioxidant studies such as the determination of total polyphenol, total flavonoids, reducing power and radical scavenging activity of the extracted yellow pigments will also be conducted on the extract. Antioxidant activity observed in this study will be additional advantage to the extracted yellow dye. The final part of this study will be focusing on identifying and characterising the three important genes which are involved in the carotenoid biosynthesis pathways namely lycopene-epsilon cyclase (*LcyE*), lycopene-beta cyclase (*LcyB*) and phytoene synthase (*Psy*). The characterisation of these genes will allow better understanding for future gene manipulation works to be conducted.

The research questions in this study are:

1) Do the local varieties of *I. batatas* leaves contain the same level of lutein and β-carotene pigments and how can they be distinguish morphogically?

2) What are the optimum extraction conditions to maximise the extraction of yellow pigments from the leaves?

3) Are the stability of the extracted yellow pigments affected by the different storage durations, temperatures and matrices?

4) What are the compositions of the yellow pigments extract and are they a good source of antioxidants?

5) Do the genes involved in the biosynthesis pathway of the leaves have similarity with the sequences from other plants?

Hence, the objectives of this study are:

1) To distinguish the different varieties of *I. batatas* through morphological variations of the leaves and storage roots,

2) To screen and determine the level of chlorophyll *a*, chlorophyll *b*, lutein and β-carotene in the different varieties of *I. batatas* leaves and to choose the best storage temperature for the leaves prior to extraction,

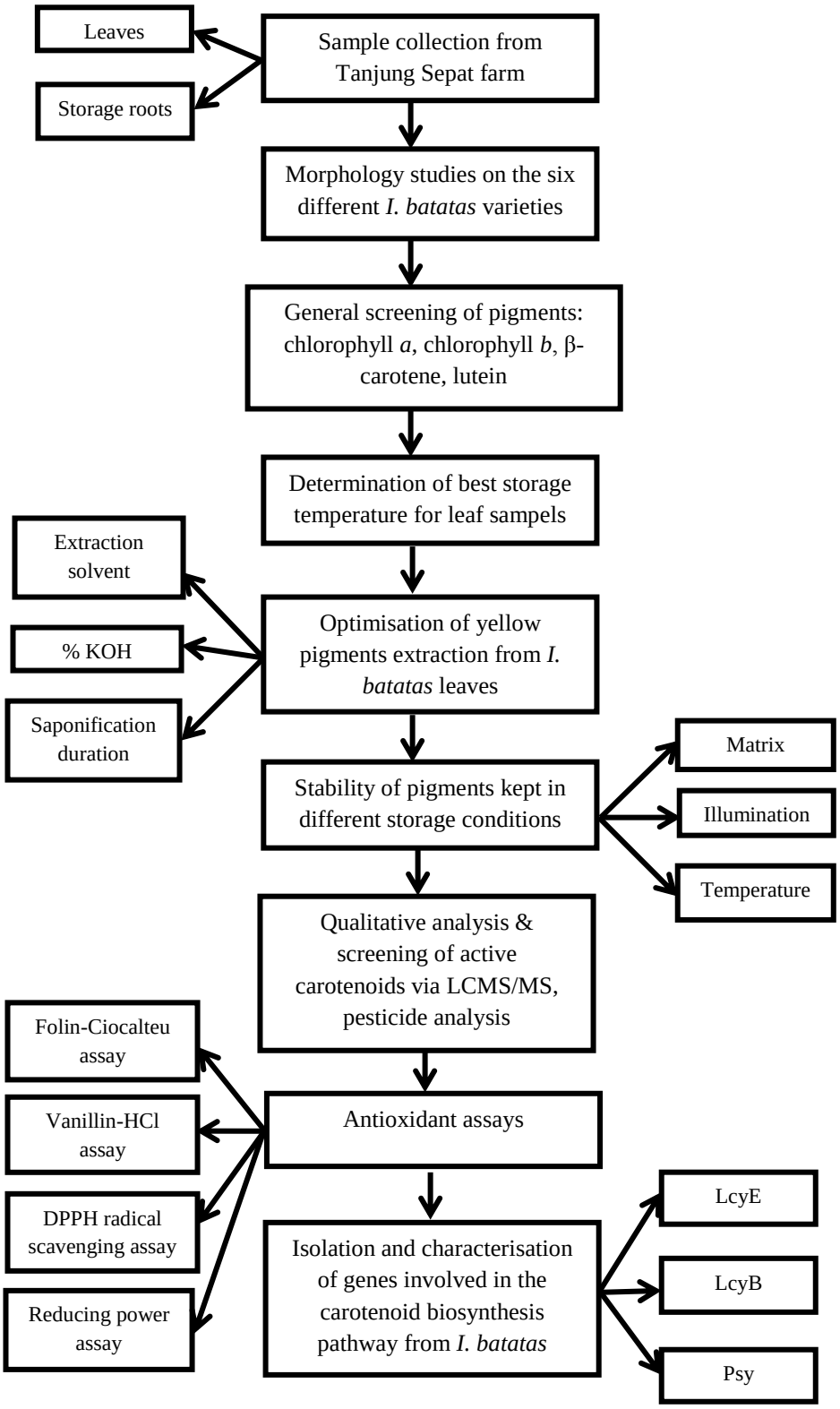
3) To identify the optimum extraction conditions for lutein and β-carotene from *I. batatas* leaves and to determine the stability of these pigments under different storage conditions,

4) To identify the composition of the yellow extract through liquid chromatography mass spectrometry / mass spectrometry method and to detect the presence of pesticides residues in the extract,

5) To determine the total polyphenols, total flavonoids, reducing power and radical scavenging activity in the yellow pigments extract from *I. batatas* leaves,

6) To identify and characterise lycopene-epsilon cyclase (*LcyE*), lycopene-beta cyclase (*LcyB*) and phytoene synthase (*Psy*) gene sequences from the *I. batatas* leaves.

FLOWCHART OF RESEARCH



CHAPTER 2

Literature Review

2.1 Introduction to *Ipomoea batatas* (Sweetpotato)

Ipomoea batatas is the sixth most important food crop in the world and ranked seventh in the total world food production after wheat, maize, rice, potato, barley, and cassava (Islam, 2006). *I. batatas* or commonly known as sweetpotato originated from the Central America and Mexico and has been dispersed worldwide due to its high yield potential and wide adaptability. Carolus Linnaeus was the first to give the scientific term *Convolvulus batatas* to the sweetpotato plant in 1753, referring to the plants grown in George Clifford's garden in the Netherlands.

Ipomoea batatas is a perennial herbaceous dicotyledonous species and is categorised under the *Convolvulaceae* family, which includes the morning glory (*I. purpurae*) plant and can be further divided into varieties or cultivars. From the group of approximately 50 genera and more than 100 species in this family, *I. batatas* is the only group that has major economic importance as food (Woolfe, 1992). Besides *I. batatas*, *Ipomoea aquatica* (known locally as Kangkong) is also grown for human consumption and eaten as dishes in China and Malaysia. The increased numbers of *Ipomoea batatas* varieties took place due to selection by human for domestication. *I. batatas* is cultivated in more than 100 countries and is an extremely important food crop in developing countries (Wang, 2005). Besides that, the *I. batatas* are also well adapted to tropical areas where high proportion of poorest people in the world lived.

I. batatas (sweetpotato), unlike *Solanum tuberosum* (potato) are not tuber propagated. A tuber can be defined as short, thickened-stem of an underground branch (Kakaty *et al*., 1992). In contrast, *I. batatas* produces primary fibrous roots, pencil roots and storage roots and they lack of merismatic buds. The storage roots are attached to the stem by a stalk of thinner roots that is usually initiated at the stem node just below the soil line. Worldwide currently, there are over 6000 varieties of *I. batatas* and they are basically distinguished by skin colour, flesh colour, and some, by origin (ICP, 2006). The skin colour of *I. batatas* storage roots typically range from white to brown to red-orange while the flesh colour may be red-orange, orange, yellow or white (more common). Besides that, the flesh of the storage roots can be either soft or firm. Figure 2.1 showed the storage roots being harvested from the *I. batatas* plant. The photograph was taken from an *I. batatas* farm located in Tanjung Sepat, Kuala Langat, Selangor, Malaysia.

Fig 2.1 The *Ipomoea batatas* plant (Location: Tanjung Sepat, Malaysia)

2.1.1 Distribution and Growth Habitat of *Ipomoea batatas*

Scientists believed that *Ipomoea batatas* was domesticated more than 5000 years ago. Recent evidence showed that *Ipomoea batatas* was originated from Central-America although contradicting reports claimed that it was from South America (Figure 2.2) (Natural History Museum, 2007). *Ipomoea batatas* was widely established in the Americas by the time Europeans first arrived there and was spread to the Old World through various routes. Due to its hardy nature and broad adaptability, *Ipomoea batatas* successfully spread through Asia and Africa during the 17th and 18th centuries. Figure 2.2 showed the route of *Ipomoea batatas* dispersion from Central America to the other parts of the world.

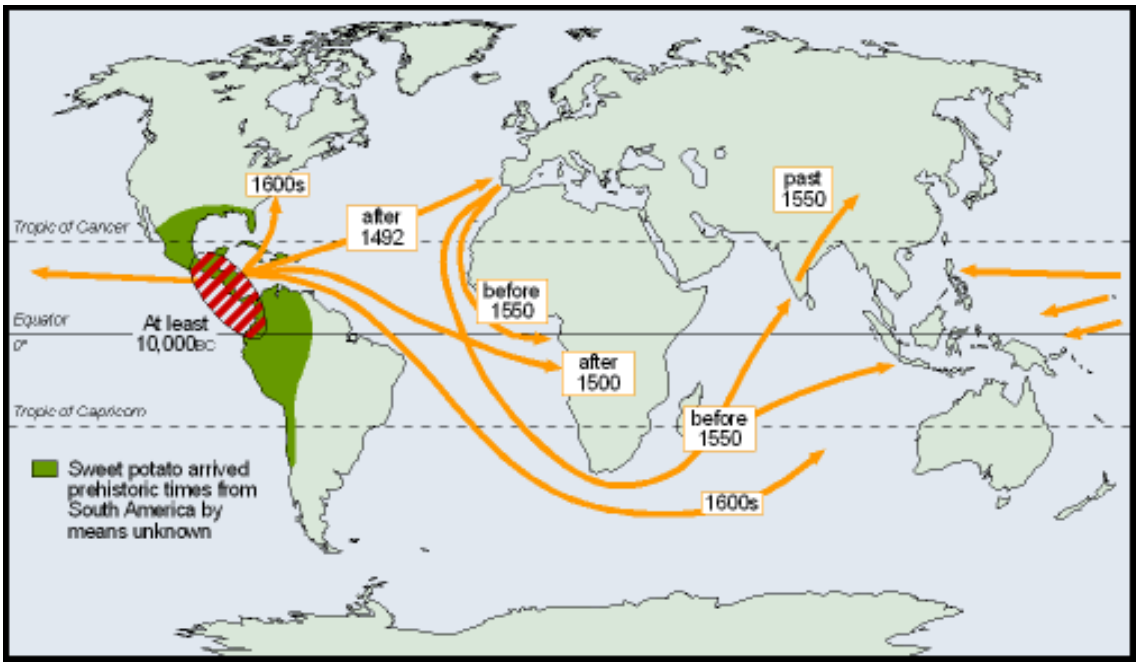


Fig 2.2 The route of *Ipomoea batatas* dispersion (Adapted from Natural History Museum, 2007)

According to FAOSTAT data, approximately eight million hectares of land were harvested resulting in approximately 107 million tons of *Ipomoea batatas* roots from more than 100 countries in the year 2010 alone (FAOSTAT, 2012). Asia is the world's largest sweetpotato producing region with an annual production of approximately 88 million tons and China contributes about 80% of this amount. Nearly half of these productions were used as animal feed while the remaining was used for human consumption. In contrast, the African region produced about 14 million tons of *Ipomoea batatas* roots annually but most of this crop is cultivated for human consumption (FAOSTAT, 2012). Previous research on *Ipomoea batatas* has been focussing on the yield and nutrient improvements in the storage roots although both the *Ipomoea batatas* roots and tops possess a variety of compounds which are beneficial to human health.

2.1.2 Classification and Taxonomy of *Ipomoea batatas*

The long-term cultivation and selection of *Ipomoea batatas* produced many different varieties with different roots skin colours, flesh colours and shapes. Variations can occur both naturally as a result of mutations which will be termed cultivar or through hybridisation by human (known as varieties) (Morton, 1981). Through the advances in biotechnology, *Ipomoea batatas* roots have been developed for special purposes such as for their high protein, starch or β-carotene contents.

Ipomoea batatas varieties are usually distinguished by the size and colour of their fruits. The examples of *Ipomoea batatas* varieties are Kotobuki (Japanese), Georgia Jet, Fernandez, Red Jewel and Okinawa. *Ipomoea batatas* can be planted for either their roots and/or forage production, in which yields often depended on the different climate, season and soil conditions (Woolfe, 1992; Hartemink *et al*., 2000; Antia *et al*., 2006).

The classification of the *Ipomoea batatas* plant is shown in Figure 2.3. *Ipomoea batatas* is located in the plant kingdom and belongs to the '*Convolvulaceae*' family, *Ipomoea* genus and *batatas* species and thus termed *Ipomoea batatas*.

While the current method of identification focussed on the variations of storage roots to distinguish the *Ipomoea batatas* varieties, it was found to be time consuming and required the formation of the roots before the different varieties can be identified. Therefore, the leaf of the plant was subsequently used as a new way to distinguish between the different varieties. This method is an economical way to overcome the drawbacks of the previous identification method. Leaves are commonly distinguished based on parameters such as length, width, shape, margin and venation. McEwen (2004) pointed out the variation in the *Ipomoea batatas* leaves in terms of shape namely cordate shaped with heavily divided single lobed and palmate shaped with seven lobes or more.

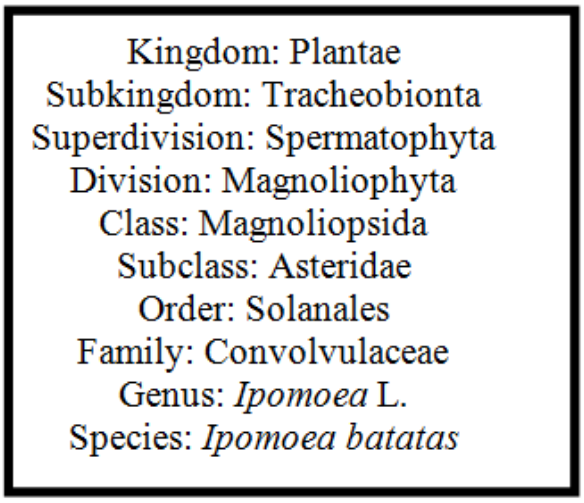


Fig 2.3 Taxonomy of the *Ipomoea batatas* (Adapted from United States Department of Agriculture)

2.1.3 Growth and Usages of *Ipomoea batatas*

Ipomoea batatas is considered to be a small farmer crop but grow well under many farming conditions. The advantages of growing this crop include the following: it has a relatively few natural enemies, it requires little or no pesticide, it can be grown in soil with little fertilizer, it has a high tolerant to weeds and is easy to plant compared to other tropical leafy vegetables (Islam, 2006). Furthermore, *Ipomoea batatas* can be grown throughout the year and therefore provide a continuous supply to industries.

Ipomoea batatas root is a rich source of carbohydrate and therefore can help curb famine when there is a rice shortage crisis. In some countries, excess storage roots are used to feed poultry besides human (Ruiz *et al*., 1980). The storage roots can be harvested within 3 to 6 months and are well adapted to warm tropical lowlands. The *Ipomoea batatas* can be propagated in two ways: by tuber and cuttings. Although naturally resistant to pest, destructions by sweet potato weevil and viruses diseases are the most common problem encountered in *Ipomoea batatas* plantation (CGIAR, 2005). *Ipomoea batatas* leaves contained high amount of pro-vitamin and was found to be on par with cassava and green leafy vegetables, hence owing them the term "pro-vitamin rich vegetable". Modern biotechnology has been used to produce varieties with a high level of pro-vitamin that can have measureable effects on both human health and nutritional status (Kimura *et a*l., 2005).

With the recent advancement in the field of biotechnology, *Ipomoea batatas* are currently used in Africa to combat Vitamin A deficiency that causes blindness and death of 250 000 to 500 000 African children annually. From this total, about two third of these children suffered from xerophthalmia, and die within a year after losing their

sight. Although *Ipomoea batatas* was a staple food for a long time in Africa, their storage roots, which mostly white flesh, contains no β-carotene which is an important precursor of Vitamin A (CGIAR, 2005).

2.1.4 *Ipomoea batatas* (Sweetpotato) Leaves

Ipomoea batatas leaves have been studied in many ways, mainly the total crude protein, crude fat, crude fibre, ash and carbohydrate contents in the leaves but little study has been done on the leaf pigments. *Ipomoea batatas* plant was once described by Longe (1986) as herbaceous creeping plant with smooth, lightly moderate green leaves and sometimes comes with a purple pigmentation along its veins. Figure 2.4 showed the leaves of *Ipomoea batatas* plant.

Fig 2.4 *Ipomoea batatas* leaves (Location: Tanjung Sepat, Malaysia)

While *Ipomoea batatas* storage roots are a good source of carbohydrates, the leaves contain additional nutritional components such as vitamin B, β-carotene, iron, calcium, zinc, and protein in much higher concentrations compared to other commercial vegetables (Islam, 2006). Some studies have reported the potential usage of *Ipomoea batatas* leaf, stalk and stem as food source particularly in developing countries (Ishida *et al*., 2000). In addition to that, their annual yield is much higher than many other green vegetables because *Ipomoea batatas* leaves can be harvested several times a year (Islam, 2006).

In a study conducted by Khachatryan *et al*. (2003), lutein was first extracted from *Ipomoea batatas* leaves to be used as a potential source of fortification in food to prevent the onset of age-related macular degeneration (AMD). The leaves of the 'Suioh' variety, which was developed mainly for its lutein content in the leaves showed the average lutein concentration of 36.8 mg/100g FW. The lutein concentration in *Ipomoea batatas* was found to be higher than *Ipomoea aquatic*a leaves (Kangkong) (11.9 mg/100g FW) as well as other fruits and vegetables as described previously by Mangels *et al*. (1993). This thus showed that the *Ipomoea batatas* leaves have the potential to be a source of lutein extraction.

Besides being used for human consumption, the *Ipomoea batatas* leaves also serve as fodder and browse for cattle, sheep, goats, pigs, and other domestic animals (Antia *et al*., 2006). The consumption of *Ipomoea batatas* leaves as vegetables is however hampered by the fact that it is considered a poor man's vegetables, coupled with the fact that it had always been used traditionally as feeds for domestic animals (Antia *et al*., 2006). The nutritional value of these leaves is gaining recognition, as the understanding between diet and health increases. *Ipomoea batatas* leaves with their high nutritive value and antioxidants may become an excellent leafy vegetable (Islam, 2006).

2.1.5 The Future of *Ipomoea batatas* (Sweetpotato) Plant

Currently, the Consultative Group on International Agricultural Research (CGIAR) is conducting researches focusing on the improvement of *Ipomoea batatas* because research on this crop is considered to be scarce compared to other crops. International Potato Center or CIP in Peru have also found that there is ample potential to expand the *Ipomoea batatas* as a valuable source of income and food and can create jobs in developing countries (CGIAR, 2005). In addition to that, the collaboration between the Centre for Potato (CIP) and the International Genetic Resources Institute (IPGRI) has come out with a plan to enhance collaboration between sweetpotato workers in Asia and Asia Network on Sweetpotato Genetic Resources which constituted of 11 partner countries (Rao and Campilan, 2001). This research has also focussed on the establishment of *Ipomoea batatas* germplasm for genetic diversity in varietal improvement.

Malaysia also contributed ample effort to transform *Ipomoea batatas* into raw food material for industries besides focussing on improving the current varietals to increase the total mass production of these storage roots (Tan and Mohammad, 2001). In China, on-going activities are conducted for *Ipomoea batatas* genetics conservation and *ex-situ* conservation using both the *in vitro* and cryopreservation techniques. Research conducted by the International Potato Centre (CIP) in China has shown that sweetpotato yield can be increased by 30-40% with the development of virus free cutting. If successful, this could save up to US $1.5 billion if this technology is used in all China sweetpotato growing regions (ICP, 2006).

With regard to medicine, the usage of the *Ipomoea batatas* variety ‘Caiapo’ has shown to increase insulin sensitivity and improve metabolic control of test subjects with diabetes type 2. Besides that, LDL cholesterol level in the male Caucasian test subjects was found to decline with the administration of *I. batatas* extract compared to placebo (Ludvik *et al*., 2000). The presence of essential oil components in the *I. batatas* leaves has also been studied using gas chromatography and mass spectrometry techniques. Characterisation of these compounds can possibly be a breakthrough in making the leaves a potential source of essential oil in the future (Wang *et al*., 2010).

All in all, the future of *Ipomoea batatas* plant looks bright, looking at the continuous conservation efforts that are conducted worldwide. Different approaches are developed to sustain the *Ipomoea batatas* plant and hopefully more research can be conducted on this humble crop benefit to mankind.

2.2 Plant Secondary Metabolites

Metabolites are organic compounds that are produced by plants and can be categorised into primary metabolites and secondary metabolites. Primary metabolites produced by plants are virtually similar for all plants, which include carbohydrates, fats, proteins, vitamins and mineral nutrients essential for the plants sustainability and the lack of these metabolites may lead to the sudden death of these plants (Croteau *et al*., 2000). Besides, they also constitute components from the Krebs cycle and the glycolysis process.

Unlike the primary metabolites, plants produced more than 30000 different types of substances which are included in the "Plant Secondary Metabolites" category (Seigler, 1998). Secondary metabolites in plants can be categorised based on their nitrogen composition, chemical structures such as the presence of rings structure and sugar, solvents solubility, or their synthesis pathways (Agosta, 1996). The pathways involved in the production of these substances are derived from the primary metabolites route. However, the amount of secondary metabolites produced is less than 1% of the total carbon amount in the plant and these substances are often stored in the plant cells or organs (Bourgaud *et al*., 2001).

Generally, plant secondary metabolites are not directly involved in the normal growth, reproduction and development of the plant. However, the absence of some plant secondary metabolites can cause long-term impairment to the organism survivability and aesthetics while some do not cause any significant change at all (Wink, 2003). The production of plant secondary metabolites are very much dependant on the cytological and morphological changes that occur in the plant. The family, genus or species of a particular plant usually produce a combination of secondary metabolites, which can be

useful for taxonomic classification (Agosta, 1996). Derivatives and minor compounds usually exist alongside the major compounds in plants. Some secondary metabolites tend to present in the active state while some are only activated upon infection, wounding or when ingested by herbivores (Wink, 2003). The production of plant secondary metabolites however is affected by the different plant genotypes, seasonal and diurnal variation and phenotypic differences between the growth developmental stages. Hence, thorough screenings of these metabolites across the different developmental stages are important to determine the highest desired compounds (Verpoorte, 1998).

A plant's ability to produce secondary metabolites is important in the plant-herbivore interaction because this shows that the plants have selective advantage compared to others (Seigler, 1998). While plant secondary metabolites contribute extensively in industries as flavouring agents and perfumes, some of these compounds are physiologically active and can be used as medicinal agents, insecticides or biological probes (Seigler, 1998).

2.2.1 Types of Plant Secondary Metabolites

The common plant secondary metabolites in nature are carotenoids, phytosterols, saponins, glucosinolates, flavonoids, protease inhibitors, terpenes, phyto-estrogens, sulphites and phytic acid and these compounds are usually classified according to their biosynthetic pathways. The three main families of compounds are: phenolics, terpenoids and alkaloids (Walton and Brown, 1999).

Phenolics are very stable plant secondary metabolites that are characterised by the presence of a benzene ring and one hydroxyl group (-OH). They are the most abundant secondary metabolites in plants and can be converted into lignin, which is the main phenolic polymer in plants. Phenolic compounds in plants include non-soluble compounds such as condensed tannins, lignins, cell-wall bound hydroxycinammic acids, and soluble compounds such as phenolic acids, phenylpropanoids, flavonoids and quinones (Rispail *et al*., 2005). Flavonoids, the pigments that constitute the red, violet and blue colour in plant is of particular interest due to their multiple roles in plants (Harborne and William, 2000). They are important in inhibiting the growth of viruses and bacteria, protecting the cells from damages by free radicals, repressing inflammation as well as protecting human against cancer and heart attack (Dixon, 1999). The examples of phenolics compounds are shown in Figure 2.5.

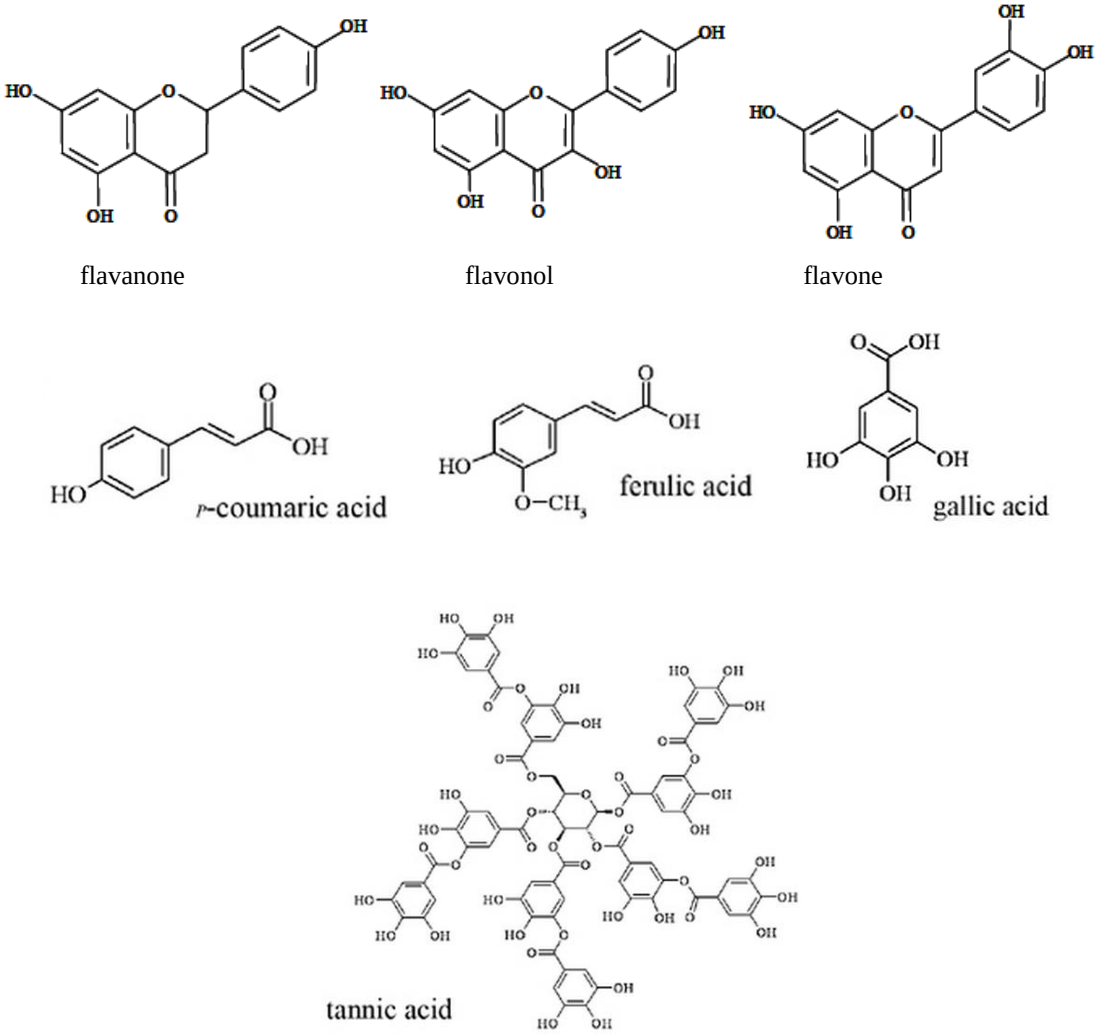


Fig 2.5 Structures of phenolics compounds (Adapted from Aoki *et al*., 2000 and Chrzanowski *et al*., 2007)

Terpenoids are synthesized from the universal five carbon precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) and consist of entirely hydrogen and carbon. They play an important role in plant-insect, plant-pathogen and plant-plant interactions (Dudareva *et al*., 2004; Paschold *et al*., 2006). The volatile terpenoids are represented by mainly isoprene (C_5), monoterpenes (C_{10}) and sesquiterpenes (C_{15}), which constitute the largest class of plant volatile compounds (Nagegowda, 2010). Currently more than 23000 individual terpenoids have been identified from higher plants and they are normally present in vegetative tissues, flowers and sometimes in roots (Köllner *et al*., 2004; Dudareva *et al*., 2004).

One of the most important groups in the plant secondary metabolites is the large group of carotenoids. Carotenoids are terpenoids which contribute mostly to the red, orange and yellow colour in fruits and vegetables (Setiawan *et al*., 2001). Carotenoids are rich in antioxidant activities and have been reported to prevent cancer and reduce the risk of heart attack (Bendich, 1993; Kumar *et al*., 2002). Green vegetables such as broccoli, spinach and kale are good source for carotenoids. Phytosterols is another type of terpenoid that are usually found in soya beans, sunflower seeds, nuts and sesame. These metabolites are chemically similar to cholesterol and have been documented to protect against colon cancer and lower cholesterol level by competing with cholesterol to be absorbed by the human body (Ostlund, 2002). Saponins, on the other hand are usually found in legumes and used as flavour additives. Besides, saponins has been reported to lower down the cholesterol levels in the bloods as well as enhancing the human immune system (Shi *et al*., 2004). The examples of terpenoids are shown in Figure 2.6.

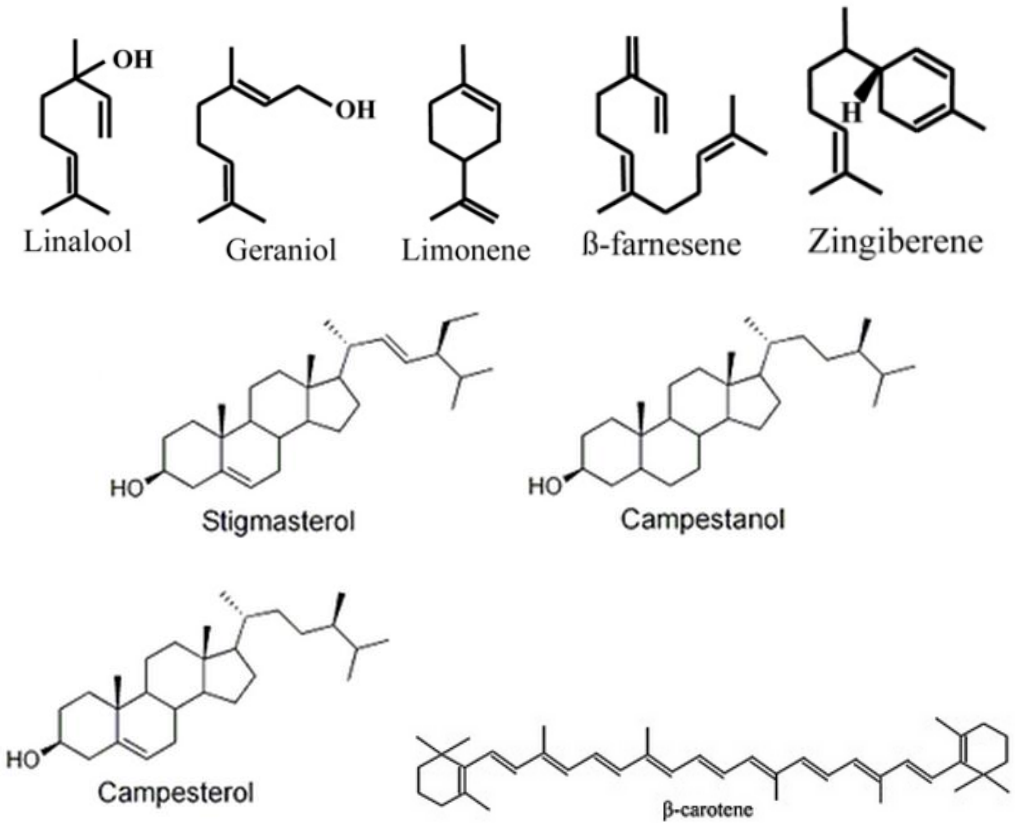


Fig 2.6 Structure of terpenoids compounds (Adapted from Moreau *et al*., 2002 and Nagegowda, 2010)

The last group of secondary metabolites in plants are the alkaloids, which are derived from amino acids and include the nitrogenous compounds. Alkaloids usually contain one or more rings of carbon atoms with a nitrogen atom in the ring. More than 10000 different alkaloids have been documented from over 300 plant families (Raffauf, 1996). They are usually found in legumes as a result of symbiotic relationship between these plants with the *Rhizobia* species in the root nodules (Wink and Waterman, 1999). The examples of alkaloids compounds include indole alkaloids, pyridine alkaloids, tropane alkaloids and quinolizidine alkaloids. The examples of alkaloids compounds are shown in Figure 2.7.

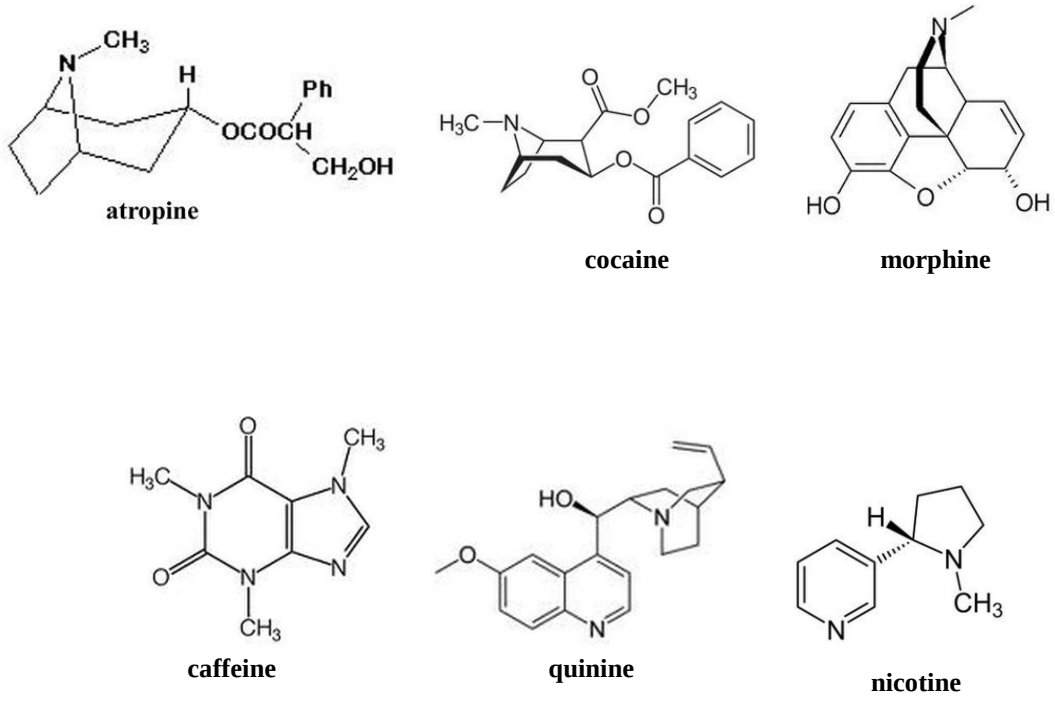


Fig. 2.7 Structure of alkaloids compounds

2.2.2 Roles of Plant Secondary Metabolites

Plant secondary metabolites can help to protect plants against their natural enemies such as fungi, worms, viruses, bacteria and many herbivorous animals since plants are immobile and therefore cannot avoid these enemies (Wink, 1999). Although physical modification such as cuticle layer is present in plants, this waxy layer alone is not enough to defend the plants from their enemies. Hence, protective tissues such as periderm are form to produce secondary metabolites to protect the plant from herbivores (Wink, 2003). It has been found and documented that compounds such as alkaloids, terpenes, saponins and tannins produced in plants are in fact allelochemicals, the chemical defence compounds that help to protect the plants from their enemies (Wink, 2003).

Secondary metabolites are also an important trait in ornamental plants and food plants as they contribute to the taste, colour and scent of the plants. Besides that, plant secondary metabolites such as alkaloids, anthocyanins, flavonoids, quinones, lignans, terpenoids and steroids are gaining commercial value as fragrance, flavouring, insecticide, dye and drug (Verpoorte *et al*., 2002). Although insects and herbivores can cause damage to plants, they are needed for pollination and seed dispersion of the plants (Harborne and Baxter, 1993). Secondary metabolites such as fragrant monoterpenes in plants are used to attract the pollinators and this characteristic made them suitable to be used in the scent industry. In addition, secondary metabolites also function physiologically by transporting toxic nitrogen out of the plant and acting as UV-protectants (the phenolics) (Harborne and Baxter, 1993). Besides playing roles in signalling and as antioxidants, secondary metabolites such as anthocyanin and carotenoids also have the potential to be made as natural colourants (Novafeel, 2005).

The productions of toxic secondary metabolites in plants are induced when the plant is attacked by herbivores or pathogens and thus serve as part of the plants' defence system against other species (Schafer and Wink, 2009). The common mode of action includes the release of some compounds into the air when the plant is attacked by insects and in return, these chemicals will attract parasites and predators to kill the herbivores. Secondary metabolites are also used to suppress the growth of neighbouring plants and the production of these defensive substances are different inter or intra species (Meijden, 1996). However, it is not clearly understood how some plant such as the nicotine plant are free from insect herbivory while not producing any secondary metabolites.

On the other hand, due to their function as a part of the plant defence system, the intake of secondary metabolites might cause fatality. For instance, the consumption of alkaloids can blocks the body ion channels, interferes with the neurotransmission and can lead to hallucinations and death (Wink, 2000). Besides that, ingestion of some phenolics can have effects on the digestion system, through the interference with enzyme activity and cell division (Wink and Schimmer, 1999). It was documented that some herbivores and plant pathogens have the ability to modify the plant metabolites and thus show the evolutionary associations between plants and pests. Some herbivores are also able to store the plant's toxin and uses it as defence chemical against their enemies (Wink, 1993).

Secondary metabolites have also gain importance in the pharmaceutical industry. These compounds contributed widely to the field of ethnopharmacology. The designs of some drugs in western medicine are based on the traditional usage of the drugs found naturally in plants. Drugs such as atropine, morphine and quinine were isolated as pure compounds from traditional medicinal plants (Verpoorte, 1998).

Plant secondary metabolite can have a number of protective functions in human such as boosting the human immune system, protecting the body from harmful free radicals and killing pathogenic germs (Dixon, 2001). Human diet with high content of secondary metabolites may help to protect the body against cancer and cardiovascular diseases (Novefeel, 2005). While secondary metabolites are known to be good for human health, how and to what extent does it affect human health is still unknown.

2.2.3 Qualitative and Quantitative Analyses of Plant Secondary Metabolites

Various methods have been developed to extract secondary metabolites from plants for characterisation and in-depth study. The usage of solvents is a common method in extraction and the types of solvent used can affect the quantitiy and types of compound extracted. Generally, two schemes are employed in the extraction of metabolites, firstly using solvents with high selectivity for certain group of compound and secondly using general solvents such as non-polar solvent (eg. petroleum ether) or highly polar solvent (eg. methanol) (Schripsema *et al*., 1996).

On the other hand, the study of plant secondary metabolites has gained popularity due to the new advances in the combined field of analytical chemistry, enzymology and pharmacology. For instance, minimal quantities of pure compounds are needed compared to previous analysis when using mass spectrometry and NMR spectroscopy techniques (Croteau *et al*., 2000). The potential new bioactive compounds extracted from plant sources are usually subjected to chemical screening and biological or pharmacological targets at the same time. The development of hyphenated techniques such as liquid chromatography/ ultra violet detection (LC/UV), liquid chromatography/

mass spectrometry (LC/MS) and liquid chromatography/ nuclear magnetic resonance (LC/NMR) with centralised acquisition of spectroscopic data can provide structural information and possible de-novo structure prediction of the secondary metabolites within a short analysis time (Jayawickrama and Sweedler, 2003).

In addition, liquid chromatography/ mass spectrometry (LC/MS) technique is also used to characterise unknown compound in an extract (Wolfender *et al*., 1998). When the LC/MS system is coupled with the usage of atmospheric pressure ionisation techniques (ACPI or ESI), the results generated are further enhanced. Furthermore, complementary and extra information on the structure of the secondary metabolites can be generated by liquid chromatography-mass spectrometry/ mass spectrometry technique. Chemical screening or metabolite profiling method is useful to distinguish the already known compounds and new molecules directly in the crude plants extract (Hostettmann *et al*., 2001). However, metabolite profilling in crude plant extract is not easy since natural products have very important structural diversity, which takes into account the atoms and stereo-chemicals orientations.

Generally, the usage of the different techniques mentioned above is dependable on the objective of the study as well as the product to be determined. With the advancement in technology, hyphenated techniques (combination of two or more analytical techniques) are used widely to substitute single conventional analytical method such as high performance liquid chromatography (HPLC), gas chromatography (GC), nuclear magnetic resonance (NMR) and mass spectrometry (MS). This is because, hyphenated techniques have higher tolerance for compounds in an enriched mixture that cannot be separated easily and the unstable state of the natural products (Bhattacharya *et al*., 1995; Wolfender *et al*., 2003).

2.2.4 Future of Plant Secondary Metabolites

The commercialisation process involving secondary metabolites is always caught in a bottleneck situation as many problems are encountered throughout the entire process. The natural route for the production of secondary metabolites in plants involves a long biosynthetic pathway involving many genes. Hence, the viability of the plant cells used is of major concern in the industry to ensure the continuous production of the desired secondary metabolites (Bourgaud *et al*., 2001). In addition, most of the secondary metabolite production requires the usage of bioreactors, which ultimately increase the cost of production and thereby making the production of these secondary metabolites not economical for industrial applications (Verpoorte *et al*., 1999). Besides that, the production of some costly metabolites might be feasible when grown in small amount but cannot be produced in massive size plant cell cultures (Verpoorte *et al*., 2002). Hence, on-going research is conducted to find cost effective strategies such as the invention of low-cost bioreactors and sterility process in production to boost the secondary metabolites industry (Bourgaud *et al*., 2001).

Besides that, the production of secondary metabolites in plants is sometime hampered by the restriction to a certain genus or species or the products are only formed during a particular growth and developmental stage or under stress or nutrient availability conditions (Berlin, 1986). In addition, some plants are hard to cultivate and collection of the wild can risk extinction of the plants while slow growing plants such as Cinchona trees requires at least 10 years of growing (Verpoorte *et al*., 2002). Various researches have been focussing on plant cell cultures as a possible method for plant secondary metabolite production in industry level. Unfortunately, in many cases, the product of interest is produced in low amounts even though extensive studies have been conducted to optimise the cell growth in the production media. Hence, this often hampered the

usage of plant cell cultures in industry (Alfermann and Petersen, 1995). There are some exceptions observed with the successful production of shikonin, taxol, berberine and ginsenoside using industrial feasible process (Buitelaar and Tramper, 1992).

Molecular biology techniques also offer new approaches to study important genes along the production pathway but they have limited applicability for secondary metabolites. This is because the synthesis of single secondary metabolites usually involved multiple gene families, and hence multiple gene transformation and coordination of gene transformation must be taken into consideration (Bourgaud *et al*., 2001). The isolation of enzymes and subsequent cloning of the encoding gene has proven to be a good way to identify the steps involved in the biosynthetic pathway (Yun *et al*., 1992). On top of that, metabolic engineering technique in the regulation of biosynthesis has been widely studied in the recent years to increase products formation in cell culture, plants and microorganism (Verpoorte *et al*., 2002).

All in all, the breakthrough in the industrialisation and commercialisation of plant secondary metabolites are indeed fascinating. However, further research has to be conducted to ensure that high quality and effective natural products can be produced from plants.

2.3 Carotenoids

Plant carotenoids constitute the red, orange and yellow lipid-soluble pigments and are embedded in the chloroplasts and chromoplasts membranes of the plant cells. Their colours are often masked by the darker colour chlorophyll in leaves (Eggink *et al*., 2001).

2.3.1 Nomenclature and Structures

Carotenoids can be divided into two main categories which are carotenes and xanthophyll. They consist of eight isoprenoid units, which are five carbon units (CH_2=C(CH_3)CH=CH_2) of 2-methyl-1,3-butadiene, joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule so that the two central methyl groups are in a 1,6-position relationship and the remaining non-terminal methyl groups are in a 1,5-position relationship. All carotenoids are formally derived from acyclic $C_{40}H_{56}$ structure (Pfander *et al*., 1998). Figure 2.8 shows the arrangement of the isoprene subunits to form a carotenoid structure.

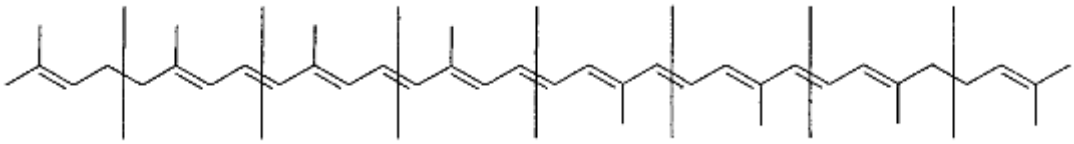


Fig 2.8 Isoprene structure in carotenoids

More than 700 carotenoids have been isolated and are mainly from plants. The different carotenoids have structures that have been modified in a variety of ways, prominently by cyclization of the end group and the introduction of oxygen functions (Britton, 1995). Carotenes refer to carotenoids that contain only carbon and hydrogen whereas xanthophylls refer to compound that contain an addition hydroxyl group or keto group or both (Britton, 1995). The major carotenoids in plants are α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin and lycopene (Kimura and Rodriguez-Amaya, 2002). Pro-vitamin A carotenoids such as α-carotene, β-carotene and β-cryptoxanthin are involved in the production of vitamin A (Edwards *et al*., 2002).

The oxygenated carotenoids (xanthophylls) are named according to the usual rules of organic chemical nomenclature with the most frequently observed are hydroxy, methoxy, carboxy, oxo, and epoxy (Pop *et al*., 2006). The cis-trans or (E/Z)-isomerism of the carbon-carbon double bonds is another interesting stereochemistry feature of the carotenoids, as the (E/Z)-isomers may have different biological properties in living organisms. Naturally occurring carotenoids are generally the (E) -isomer, although exception are known to occur such as the (15Z) -phytoene isolated from carrots, tomatoes and other organisms (Pop *et al*., 2006). However, some carotenoids can undergo isomerisation rapidly during workup, and hence many (Z)-isomers that are described in literature as natural products are actually artifacts. In general, the (Z)-isomers percentage is rather low, but enhanced at higher temperature and upon exposure to light (Pop *et al*., 2006).

2.3.2 Roles and Functions of Carotenoids

In *vivo*, carotenoids are often found in precise locations and orientations in subcellular structures and their chemical and physical properties are strongly influenced by other molecules in their vicinity, especially proteins and membrane lipids (Britton, 1995). Carotenoids produced by photosynthetic organisms, bacteria, algae and higher plants are generally located on the light harvesting pigments involved in photosynthesis and play a major role in the photoprotection of these organisms from the damaging effects of their own endogenous photo-sensitizer, the chlorophyll (Bartley and Scolnik, 1995). Hence, this feature has prompted the usage of carotenoids for the treatment of patients with photosensitivity disease (Tee, 1995). Besides that, they also function as photosynthetic accessory pigments and are essential structural components of the photosynthetic antenna and reaction center complexes (Bartley and Scolnik, 1995). Carotenoids also help to decrease the fluidity of the biological membrane and thus maintain the shape of the cell besides functioning as the precursor of abscisic acid, a phytohormone that helps to modulate developmental and stress processes in plants (Sandmann, 2002).

The growing interest in the potential health benefits of carotenoids are due to the belief that carotenoids play an important role in reducing the risk of developing some degenerative diseases. Surprisingly, this action was found not linked to the provitamin A carotenoids, and so the study and quantification focussing on provitamin A only is no longer sufficient (Kimura and Roddriguez-Amaya, 2002). Other carotenoids such as lutein, zeaxanthin, and lycopene have been reported to have antioxidant activities (Bowen, 2005). Carotenoids also function in gene activation, inflammation responses, quenchers of singlet oxygen, triplet sensitizers, inhibitors of free radicals reactions and in immune processes (as a modulator of lipoxygenases) (Bendich, 1993). Since cancer

is believed to be brought about by the reactive oxygen species and free radicals, carotenoids are seen as having the ability to fight against cancer (Kumar *et al*., 2002). Carotenoids are also important antioxidant to slow down the process of aging in human since a large part of the aging process is also due to oxidation.

Another important usage of carotenoids is as food colourants. Carotenoids from natural extracts such as annatto and saffron have been used for centuries as natural food colourants. Synthetic carotenoids are also available and are mass produced in industry, and therefore are used extensively in processed and fabricated food colouring (Bauernfeind, 1972). β-carotene was the first synthetic carotenoid to hit the market in 1954 and plays a great role as economically feasible colour varieties for fat-based and water-based foods including butter, cheese and ice-cream (Bauernfeind *et al*., 1971).

Carotenoids are important because humans are not able to synthesize them and therefore the dietary intake of food containing these phytochemicals are essential for overall well-being. It was found out that, from over 700 naturally occurring carotenoids, up to 50 of them may be absorbed and metabolised by the human body. From this group, approximately 14 have been identified in the human serum. Food such as fruits (watermelon, orange, mango etc) and vegetables (tomatoes, carrot, spinach etc) are rich sources of carotenoids for the body. Leafy vegetables are the most accessible year round sources of carotenoids worldwide (Kimura and Rodriguez-Amaya, 2002). Green leafy vegetables contain lutein (β, ε,-carotene-3, 3'-diol), β-carotene (β,β-carotene), violaxanthin (5,6,5,6'-diepoxy-5'6,5'6'-tetrahydro-β,β-carotene-3'-diol) and neoxanthin (5',6',-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro-β,β-carotene-3,5,3'-triol) as principal carotenoids (Kimura and Rodriguez-Amaya, 2002). However, the amount of

carotenoids in green leafy vegetables are subjected to natural variation, variety or cultivar, climate and stage of maturity and this may account for the occurrence of divergence and differences for the same foods (Kimura and Rodriguez-Amaya, 2002).

Although in the past β-carotene was suggested to be a good antioxidant, studies conducted later found that fruits and vegetables are actually poor sources of β-carotene but good sources of oxygenated xanthophylls, which has little vitamin A activity (Beecher and Khachik, 1984). On the other hand, vitamin A deficiency is known to cause blindness, particularly in third world countries where 250, 000 to 500,000 children are prone to blindness each year, as estimated by the World Health Organisation (WHO) (Sommer, 1995). Pro-vitamin A carotenoids such as β-carotene and lycopene in this case can be good sources of vitamin A in mammals and therefore can help to curb vitamin A deficiency.

2.3.3 Carotenes

Carotenes include provitamin A pigments with vitamin A activity which is essential for good eyesight and this group of pigments was the main focus of initial interest in this field (Cutler, 1984). β-carotene is the most important vitamin A precursors in the human nutrition and the amount exceeds the level of other vitamin A active compounds (Bauernfeid, 1972). This has led to intensive studies on β-carotene in many fruits and vegetables. However, later it was found out that the protective power that lies in these fruits are also contributed by their oxygenated counterparts, the xanthophylls (Beecher and Kachik, 1984). The structures of carotenes are shown in Figure 2.9.

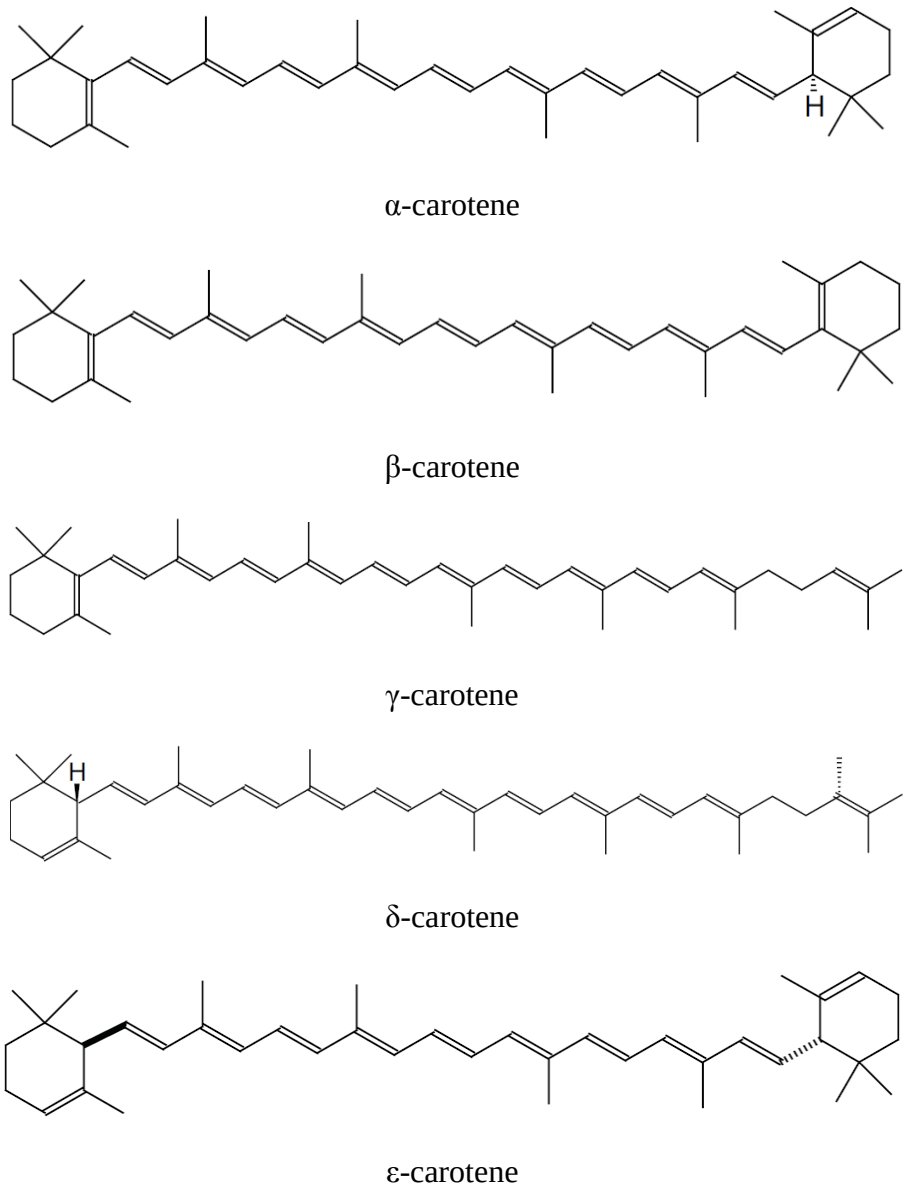
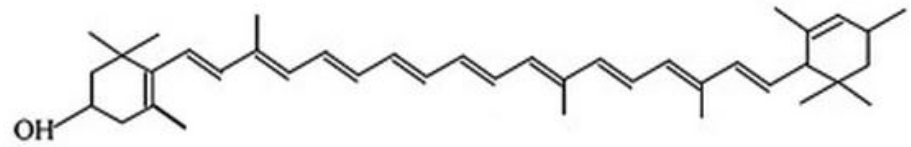


Fig 2.9 Structures of α-carotene, β-carotene, γ-carotene and ε-carotene (Adapted from Pop *et al.,* 2006)

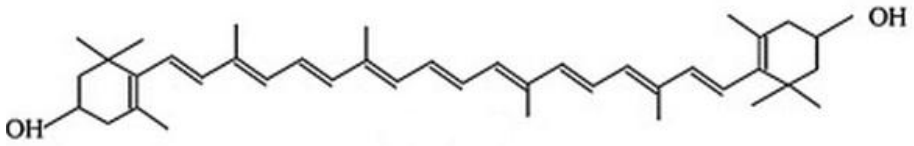
2.3.4 Xanthophylls

The two common xanthophylls, lutein and zeaxanthin are the major pigments of the yellow spot in the human retina. The major sources of xanthophylls are from green leafy vegetables and the lutein level is often higher than zeaxanthin because lutein is a major chloroplast pigment (van het Hof *et al*., 1999). Besides, lutein is also one of the five most common carotenoids found in the human diet and is readily available from green vegetables, in contrast to β-carotene (van het Hof *et al*., 1999).

Both the lutein and zeaxanthin represents approximately 36% and 18% of the total carotenoids content of the retina thereby suggesting their specific biological function in ensuring good retina function (Landrum and Bone, 2001). Besides, xanthophylls are also believed to protect human against age-related macular degeneration (AMD) (Bone and Landrum, 1992). The mechanism by which lutein and zeaxanthin help to protect the eyes is through their roles as blue light filters and/or as antioxidants (Snodderly, 1995). Xanthophylls may also be important as protective factors in retinal pigment epithelium (RPE) of the new-born infant. Besides that, the xanthophylls have been reported to be involved in the prevention of some types of cancer and cardiovascular diseases (Dweyer *et al*. 2001, Demming-Adams and Adams 2002). The structure of the xanthophylls are shown in Figure 2.10.



Lutein



Zeaxanthin

Fig 2.10 Structures of lutein and zeaxanthin

The presence of lutein and zeaxanthin in human blood and tissues is entirely due to ingestion of food sources containing these xanthophylls (Perry *et al*., 2009). Besides, it was also suggested that xanthophylls can diffuse across the placenta in a specific amount since direct correlations were observed between the respective xanthophylls level in both the mothers' and babies' plasma (Jewell *et al.*, 2000). The high intake or high serum level of lutein and zeaxanthin have been associated with lower risk of developing cardiovascular disease, several types of cancer (Granado *et al*., 2003) and cataracts (Olmedilla *et al*., 2003).

The lutein pigments have also been studied as a potential source of natural yellow dye. Currently, lutein is extracted from the African marigold (*Tagetes erecta*) which is an ornamental plant belonging to the composite family (Prateesh *et al*., 2009). Since then, the usage of lutein has diversified into cosmetics, nutritional and pharmaceutical industries.

2.3.5 Carotenoids Biosynthesis Pathway

The biosynthetic pathway of carotenoids is complex and involves numerous types of enzyme coding genes. The isolation and cloning of the genes encoding the carotegenic enzymes marked the beginning of the molecular description on the carotenoids biosynthesis in plants. Recent studies on the biochemistry of carotenogenesis provide the possibility of manipulating this pathway in crop plants for the production of a particular carotenoid. "Carotenogenesis" is often used to describe the process involved in the biosynthesis of carotenoids.

Several methods have been employed to study the genes involved in carotenogenesis. The initial study of genes started with the cloning of the phytoene desaturase (PDS) gene from cyanobacteria due to the close resemblance between the cyanobacteria and plants carotenoid biosynthesis pathway (Chamovitz *et al*., 1990). Besides that, the transposon tagging method was used in cloning the zeaxanthin epoxidase 1 (Zep1) from the *Nicotiana plumbaginigfolia* by screening for ABA-deficient phenotype while the map-based cloning had successfully cloned novel genes from tomato (Hirschberg, 2001; Marin *et al*., 1996). Carotenoids pigments are synthesized within the plastids in plants while the enzymes involved in the biosynthesis are nuclear encoded. However, recent study found that the final step in the synthesis of the ketocarotenoid astaxanthin in the green algae *Haematococcus pluvialis* took place in cytoplasmic lipid vesicles, which may provide information on the different biosynthetic pathways in the different organisms (Grünewald *et al*., 2001). Figure 2.11 showed the biosynthesis pathway of carotenoids in plants.

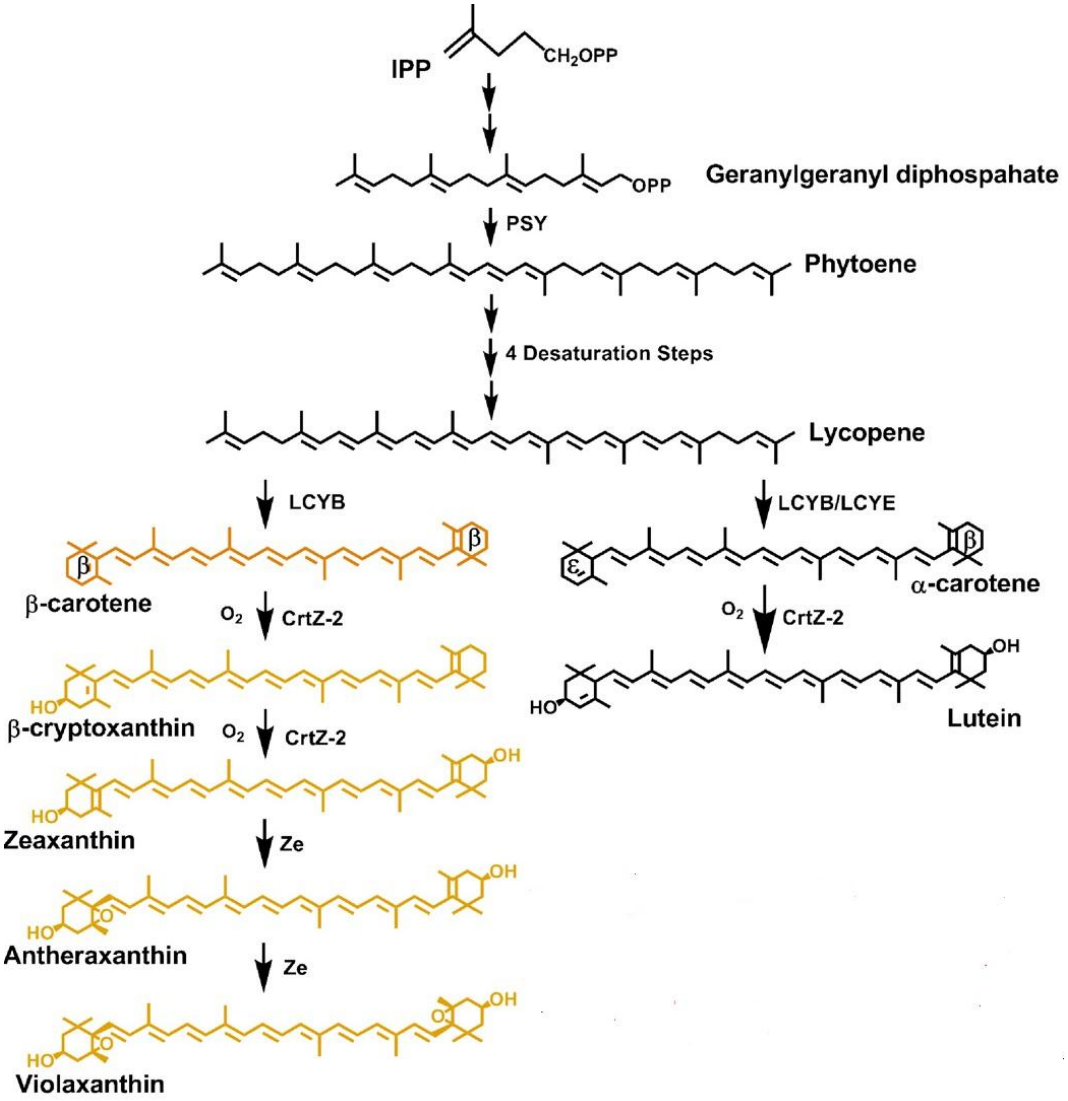


Fig 2.11 Carotenoids biosynthesis pathway (Adapted from Guzman *et al*., 2010)

Carotenoids, like other isoprenoids are built from the isopentyl diphosphate (IPP), a 5-carbon compound. IPP, is produced from pyruvate and glyceraldehyde-3-phosphate through the "DOXP pathway" (Lichtenthaler *et al*., 1997). Geranylgeranyl pyrophosphate (20 carbons) is formed by the multiple condensation reaction of IPP involving the prenyl transferase, farnesyl pyrophosphate synthase and geranyl pyrophosphate synthase enzymes. The condensation of two geranylgeranyl pyrophosphate (20 carbon chain) to form phytoene is the first committed step in the carotenoid biosynthesis cycle (Hirschberg, 2001). It was documented that two phytoene synthase (Psy) genes existed in tomato, the *Psy1* gene encoded a fruit and flower specific isoform whereas the *Psy2* encoded the isoform that was usually found in green tissues (Fraser *et al*., 1999). The phytoene synthase enzyme is the rate-limiting enzyme in flowers and fruits and therefore can be used as a key regulator in carotenogenesis.

The branching point in the carotenoid biosynthesis pathway occurs during the cyclization of the 40 carbons lycopene which divide the carotenoids into carotenes and its derivative xanthophylls. The lycopene β-cyclase helps to catalyse the formation of a two-steps reaction that creates two β-ionone rings at each end of the backbone structure (Hirschberg, 2001). On the other hand, lycopene ε-cyclase creates one ε-ring and another β-ionone ring at the different ends to give δ-carotene. The formation of xanthophylls involves the hydroxylation process of the cyclic carotenes which takes place at the 3C, 3'C positions (Sun *et al*., 1996). This process is carried out by two types of enzymes each for the β-rings and the ε-rings respectively. The β-carotene hydroxylases convert β-carotene to β-crytoxanthin and finally to zeaxanthin with the presence of iron co-factor (Bouvier *et al*., 1998). In previous study, two β-carotene hydroxylases enzymes were discovered in both *Arabidopsis* and tomato (Hirschberg, 1998). The neoxanthin pigment will continue the process in the carotenoid biosynthesis

pathway to form abscisic acid (ABA), a plant hormone. Abscissic acid or commonly known as ABA is a type of hormone produced by plants when grown under stress (eg. salt and drought stress). The production of ABA share a same pathway with the carotenoids and both play important roles in plant responses towards stress (Davison *et al*., 2002; Johnson *et al*., 2008).

In the recent years, genes involved in the carotenoids biosynthesis cycle has been isolated and cloned from many plants such as *Arabidopsis thaliana*, *Solanum lycorpersicon* (Tomato), *Brassica napus* (Rapeseed) and *Nicotanum tabacum* (Tobacco) (Giulianol *et al.*, 2008). In the future, more genes from other plants would be study to better understand the carotenoid biosynthesis pathway in plants.

2.3.6 Sources of Carotenoids

In a study conducted by Rodriguez-Amaya (1997), carotenoids are categorised amongst the most abundantly found micronutrients in vegetables and fruits, which contributed up to approximately 82% of the vitamin A nutrients in developing countries. Results also showed that mango, papaya, guava, persimmon, prune, tomato, lettuce, sweet potato and dill are amongst the rich sources of carotenoids (Ben-Amotz and Fishler, 1998). Lutein extracted from natural sources has been approved by the Eropean Union and was given the code E161B. The market for lutein as food additive can amount up to $150 million per year in US alone (Sánchez *et al*., 2008). However, it was found that the estimated daily uptake of lutein (approximately 1.5 mg/ day) is much lower compared to the recommended level of 6 mg/day and therefore the daily requirement of lutein in human diet can be fulfilled with the biofortification of food with lutein (Johnson-Down *et al*., 2002).

Currently, the commercial source for natural yellow dye is from marigold (*Tagetes erecta* L.) flowers which are grown widely in Africa. However, due to their low lutein content (approximately 0.03% dry weight), other alternatives have been studied widely (Sánchez *et al*., 2008). Microalgae have been proposed as the suitable source for lutein extraction. Several strains of microalgae such as *Muriellopsis* sp. (Del Campo *et al*. 2001, 2007), *Chlorella zofingensis* (Del Campo *et al*. 2000) and *Scenedesmus almeriensis* (Sánchez *et al*. 2007) have been studied due to their high content of lutein. However, despite their potential as a source for lutein, both marigold flowers and microalgae requires opening of land and labour forces for plantation and management and these will without doubt increase the cost of production for lutein. Besides that, production of microalga at commercial scale also requires the usage of large photobioreactors outdoors and special culture management system to ensure optimum productivity (Sánchez *et al*., 2008). This makes the natural dye less economical and desirable to be used for industrial applications. Hence, there is a need to look for a source with high content of lutein but has lower production cost. With this objective in mind, numerous studies have been conducted on various fruits and vegetables with the hope to find a cheaper alternative for natural yellow dye. Ishiguro and Yoshimoto (2007) found that the *Ipomoea batatas* leaves contain higher level of lutein compared to others and hence, this provides an opportunity for the *Ipomoea batatas* leaves to be a potential source for lutein extraction.

2.3.7 Extraction and Stability of Carotenoids from Biological Sources

Carotenoids from fruits and vegetables have been studied extensively in the recent years and often include the usage of HPLC to separate, identify and quantify the components in the extract. However, pigment separation using HPLC will result in high cost of production and require technicians for operation. Thus, solvent-solvent extraction method is often used to extract the carotenoids from their sources. Complete extraction of carotenoids often involved several steps and a mixture of several types of solvents. This is especially true when the pigments are of different polarities found in complex matrix. Due to their hydrophobic nature, organic solvents are used to extract carotenoids.

Carotenoids extraction has been performed on several sources such as oil palm, green leafy vegetables, alfalfa, seeds, alga (*Dunaleilla*), microalgae (*Scenedesmus almeriensis*) and bacteria (*Gemmatimonas aurantiaca*) (Ben-Amotz and Avron, 1990; Sánchez *et al*. 2007; Takaichi *et al*, 2010). Different types of solvents have been used to extract carotenoids from the different sources. Solvents such as acetone (Liu *et al.* 2007; Sánchez *et al*. 2008), methanol (Wang and Liu, 2009), tetrahydrofuran (Arena *et al*. 2000) and 4 petroleum ether: 1 tetrahydrofuran (Bulda *et al.* 2008) have been widely used to extract carotenoids from different types of samples. The selection of solvents for extraction is important to reduce the formation of isomers (Pesek *et al*., 1990). For instance, the use of chlorinated solvents was found to cause isomerisation of the β-carotene pigments.

Saponification is a major step in the extraction of carotenoids from vegetables and fruits sources and was considered to be the best method to remove chlorophylls (and degradation products), “unwanted” lipids and other interfering substances and

hydrolysing carotenoids esters (Granado *et al*., 2001). However, this step may be viewed as time-consuming and involved the usage considerable number and volume of solvents during partition. Saponification can be conducted during the homogenisation process, although it is usually performed after organic extraction.

Carotenoids may produce artifacts, or undergo isomerisation and degradation due to the conditions during extraction such as temperature, hydrolysis time, potassium hydroxide (KOH) concentration (Kimura *et al*., 1990; Craft and Granado, 1993). Therefore, optimising the extraction procedure of carotenoids from a new source is essential to reduce the cost of extraction and the degradation of the products. Study by Granado *et al* (2001) concluded that saponification process is mostly dependent on lipid content, KOH concentration, time and temperature. The carotenoids extraction process also requires one or more heating steps to allomerize the chlorophyll and transforms them from green to dull olive-green pheophytin and pheophorbide as observed in spinach and parsley extraction (Schwartz and Lorenzo, 1991; Mínguez-Mosquera and Gandul Rojas, 1995).

Supercritical fluid extraction has been claimed to be a milder and more efficient method to extract carotenoids from vegetables and plant samples. Liquid-liquid extraction method used in industrial are thought to be harsher compared to laboratory methods which often leads to carotenoid degradation (William, 1984) . However, in a study conducted by Seo *et al*. (2005) on pumpkin, it was observed that the total carotenoids recovery during extraction process was almost similar between the liquid-liquid extraction and supercritical fluid extraction techniques.

Successfully extracted carotenoids are vulnerable to degradation and hence, the stability of carotenoids is always taken into consideration. The colours of the carotenoids are contributed by the presence of conjugated bonds in their structures (Taungbodhitham *et al*., 1998). The double bonds in the carotenoid structure make them more prone to degradation upon exposure to light, oxygen and acid (Chen and Chen, 1993). When the double bonds become saturated, the characteristic carotenoid colour then disappears. In addition, the enzyme lipoxygenases will also catalyse the degradation of xanthophylls (lutein and zeaxanthin) when triggered by the presence of light (Biacs *et al*., 1992). Pigments degradation in extracts is also catalysed by enzymes such as chlorophyllase and Mg-dechelatase which can be inactivated by heating and extraction with organic solvents (Ogura, 1972).

Stability study conducted on lutein and β-carotene pigments in tomato juice shown that pigment degradation occur more rapidly with increasing storage temperature and exposure to light (Lin and Chen, 2005). Continuous degradation pattern of carotenoids was also observed in other systems such as in saffron (Tsimidon and Biliadesis, 1993), papaya (Moreno-Alvarez *et al*., 2000) and plums (Gabas *et al*., 2003) with increasing temperature and storage duration. Studies conducted on carrot juice showed that the level of β-carotene, lutein and vitamin A decreased with increasing temperature and storage duration. The presence of light was also found to be more destructive to the carrot juice (Chen and Chen, 1995). Besides that, study conducted on papaya and pineapple showed that both carotenoid and anthocyanin pigments deceased progressively with the increasing blanching temperature and time (Sian and Ishak, 1991). In a study on sweetpotato, gradual decrease in the lutein contents was observed throughout the growing seasons in 13 different cultivars studied. The lutein content in

the different varieties had average decrement from 21.8 to 10.0mg/100g FW, while the content in 'Suioh' leaves decreased from 25.7 to 12.9mg/100g FW (Ishiguro and Yoshimoto, 2007).

In addition, a study conducted on the carotenoids stability of orange peel, carrot and sweet potato kept at different temperatures (4°C, 25°C dark, 25°C light and 40°C) concluded that orange peel had the highest stability with the highest carotenoids lost observed in sample stored at 40°C (93.3% carotenoids loss) after 45 days of storage. Carrot on the other hand showed the least stability with total pigments degradation observed only after 20 days of storage at 25°C and 40°C while sweet potato recorded total carotenoids degradation on the 30th storage day (40°C). Overall, increasing carotenoids degradation was observed with the increased in storage temperatures, storage durations and presence of light (Cinar, 2004). A study on *Terminalia catappa* leaves concluded that the illumination is a more determinant factor of the stability of carotenoids compared to storage temperatures (Lopez-Hernandez, 2001). Besides that, total carotenoids in cassava also showed pigments degradation with increasing storage durations regardless of varieties (Alcides *et al*., 2010). Another important factor, which is the storage medium for carotenoids also contributed to the stability of these pigments. For example, lutein pigments extracted from *Scenesmus almeriensis* was found to be more stable when kept in olive oil than in acetone (Ceron-Garcia *et al*., 2010).

Carotenoid analysis is inherently difficult and great care is needed in carrying out this analysis. Precautionary measures are necessary to avoid artifact formation and loss of carotenoids during analysis. Some of the measures used include exclusion of oxygen, protection from light, storage at lower temperatures, usage of high purity and peroxide free solvents and completion of analysis within the shortest possible time (Kimura and Roddriguez-Amaya, 2002). Despite the tedious requirements, scientists continue to

study the different sources of carotenoids and enhancement methods to extract them. Besides that, the usage of liquid chromatography/ mass spectrometry (LC/MS) technique to characterise unknown constituents is also important to correctly determine a particular compound (Wolfender *et al*., 1998). When coupled with the usage of atmospheric pressure ionisation techniques (ACPI or ESI), the results generated are further enhanced.

2.3.8 Past and Current Biotechnological Approaches in Studying Carotenoids Biosynthesis

The global market for commercially used carotenoids has risen on an average of 2.9% yearly, thereby contributing to the growth from $887 million in 2004 to $1 billion in 2005 (Marz, 2005). While most carotenoids sources are from chemical synthesis and extraction, fermentation is seen to be next "in" thing for the production of these carotenoids. A new breakthrough in carotenoids occurred after the year 2000 when lutein was found to reduce the age related macular degeneration disease and this new pharmaceutical market rose to $139 million in 2004 compared to $64 million in 1999 (Del Campo, 2007). Other carotenoids have also received a boost in their market values as more studies unveil the benefits of these carotenoids to human health.

Current studies on carotenoids have been focussing on optimising both the conventional breeding and transgenic strategies to increase the production of useful and beneficial carotenoids and add nutritional value to food products (Morris *et al*., 2004). The molecular and biochemical mechanisms that govern carotenogenesis are particularly important and have led to various researches conducted on these areas. There is also a

growing interest for genetic engineering in carotenogenesis process with the success in the cloning of the carotenoids biosynthesis genes (Giovannucci, 1999). For instance, carotenoids with the β-ring can be converted into retinol, a precursor of vitamin A and thus can improve human health. The antioxidant capacity of carotenoids and its capability to function as colourants in industrial applications have provided economic importance for these carotenoids. Moreover, successful research in the production of lycopene, β-carotene and astaxanthin from the genetically modified microorganism, *Candida utilis,* has broken the barrier of conventional production of carotenoids (Misawa and Shimada, 1998). The combinations of genes from different organisms involved in the pathways also provide the possibility of synthesising novel carotenoids in the near future. However, thorough study has to be conducted due to the specificity of the enzymes used in the pathways in the different organisms.

In addition, genetic manipulation has been carried out on *Brassica napus* (rapeseed) to increase its carotenoids production. This is performed by the overexpression of the bacterial phytoene synthase (*Psy*) gene and it succeeded with a 50-fold increment in the carotenoids level (Shewmaker *et al*., 1999). Besides, the advancement in metabolic engineering has also help to improve the nutritional value in major crops such as rice. Rice was engineered to be capable of producing β-carotene and can potentially be useful to prevent vitamin A deficiency in Asia, Africa and South America (Ye *et al*., 2000). Genetic engineering in potato was based on the overexpression of the CrtB gene in the potato tuber and resulted in the increase of β-carotene, lutein and violaxanthin levels in the tubers (Ducreux *et al*., 2005). Genetic manipulation on plant leaf was conducted to increase their resistance to stress and is based on the carotenoid photoprotective roles to

scavenge reactive oxygen species and to quench dangerous triplet states of chlorophyll (Demmig-Adams *et al*., 1996). As a result, these leaves have a higher resistant to photooxidative stress and lipid peroxidation than the wild type.

In a nutshell, the future of the carotenoids, particularly its biosynthesis process is indeed very bright especially with the on-going breakthrough approaches and the newer biotechnological techniques. Hopefully, more research can be conducted in this field to explore the other benefits and usages of carotenoids.

2.4 Antioxidants

Free radicals and reactive oxygen species (ROS) have contributed to the occurrence of many degenerative diseases such as arthritis, cirrhosis, cancer, Alzheimer's disease and aging (Abotsi *et al*., 2010; Kris-Etherton *et al*., 2002; Ames *et al*., 1993). The production of the dangerous oxygen reactive species is dependent on two factors: from exogenous factor such as tobacco smoke and secondly from endogenous factors contributed by processes occurring within the body such as normal aerobic respiration and stimulation of macrophages and peroxisomes (Adedapo *et al*., 2008).

Reactive oxygen species are dangerous because they are capable of oxidizing biomolecules in the body, causing peroxidation of the lipid membrane and cell disruption that leads to cell death (Campo *et al*., 2003). Besides, free radicals and reactive oxygen species can cause the production of excessive inflammatory mediators such as superoxide and hydrogen peroxide that can cause destruction to cartilage and synovial fluid in the human body (Ostrakhovitch and Afanas'ev, 2001). While the production of these free radicals does occur during normal body metabolism, increased level can lead to toxicity in the body. Free radicals exist in many forms, which include superoxide, hydroxyl, hydroperoxyl, peroxyl, and alkoxyl radicals (Rimbach *et al*., 2005). The examples of free raidcals are shown in Figure 2.12.

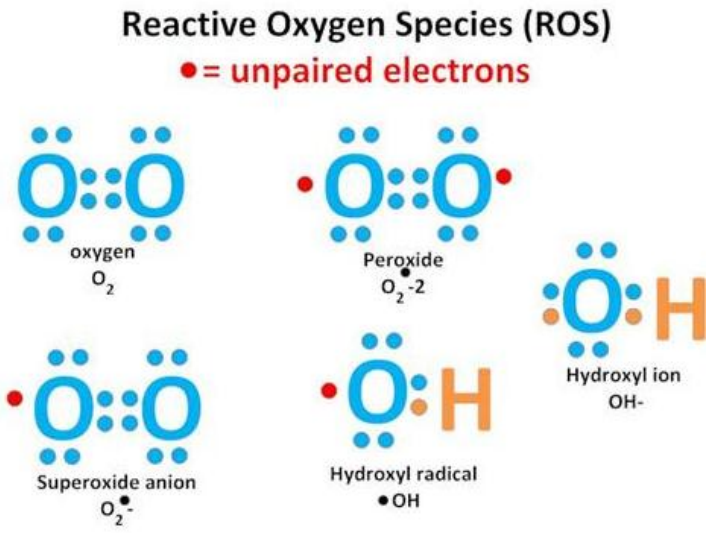


Fig 2.12 Examples of reactive oxygen species (ROS)

Free radical scavenging compounds with the ability to quench free radicals are being studied extensively. Since active oxygen species such as OH^- and O_2^- can cause oxidative damage, oxygen scavenging agents such as natural phenolics (including flavonoids and tannins) have been studied for their role in preventing cell damages (Bae and Suh, 2007). The examples of natural antioxidants that can be acquired through diet are chlorophylls, flavonoids, vitamin C, selenium and lycopene. Despite widely known as a natural antioxidant, vitamin C does not contribute to the antioxidant capacity of many fruits and vegetables (Wang *et al*., 1997). Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used in industries to preserve food or to add nutritional value to the food products. The ability of antioxidants to prevent food deterioration and to extend shelf life of the food causes it to be used widely in the food industry (Hotta *et al*., 2002). Antioxidants are also produced in our body, which include uric acid, amino acids and high density lipoprotein (HDL).

2.4.1 Benefits and Roles of Antioxidants

Most of the fruits and vegetables that we consume in our diet are rich sources of phytochemicals such as polyphenols, flavonoids, carotenoids and tannins. These compounds contained high level of free-radical scavenging activity and can help to reduce the risk of cardiovascular diseases, inflammatory joint diseases (eg. rheumatoid arthritis) and other chronic diseases (Abotsi *et al*., 2010). There are basically three main functions of antioxidants in human body: firstly to suppress the formation of free radicals and reactive oxygen species in human body which will eventually reduce the harmful effects caused by these free radicals. Secondly, antioxidants also act as scavenger (includes singlet and triplet oxygen quenchers and enzyme inhibitors), hunting down the available free radicals in the body and thirdly, as a substrate for the free radicals and reactive oxygen species in the human body (Larson, 1988; Wu and Simin, 1999). Phytonutrients from plants with antioxidant properties can prevent lipid peroxidation as well as cells ageing caused by the free radicals (Ames *et al*., 1993).

Besides that, immune cells (such as phagocytocic cells) in the human body also produce free radicals to destroy invading pathogenic microbes as a part of the normal body defense system (Droge, 2002). The imbalance between free radicals and antioxidant levels in cells would lead to the ineffectiveness of the immune system. Hence, eating fruits and vegetables rich in antioxidants can help to reduce blood pressure, reduce inflammation and detoxify contaminants and pollutants (Ascherio *et al*., 1992; Sacks and Kass, 1988). Besides, high density-lipoprotein (HDL) produced in the body also helps to inhibit the oxidation of low density-lipoprotein (LDL) thus preventing atherosclerosis and cardiovascular diseases (Salvayre *et al*., 2006).

Polyphenols are plant compounds with high level of antioxidant activity, which are mainly contributed by their redox properties. They are capable of adsorbing and neutralizing free radicals, decomposing peroxides and quenching singlet and triplet oxygen (Zheng and Wang, 2001). In addition to that, flavonoids are a large group of natural phenolic compounds and consist mainly of flavonols, flavanols and anthocyanidins. The water-soluble glycosides and acylglycosides of anthocyanins are important antioxidants among these flavonoids (Wang *et al.*, 1997; Tsuda *et al*., 1999). Anthocyanins are also an important group of antioxidants and food colourants which has also been reported to have therapeutics properties such as vasoprotective and inflammatory properties (Lietti *et al*., 1976) as well as anticancer and chemoprotective properties (Karaivanova *et al*., 1990). Carotenoid is another important group of pigments which function in gene activation, in immune processes (as a modulator in lipoxygenases) and as single oxygen quenchers (Bendich, 1993). Besides that, biochemical and epidemiological studies also found that carotenoids may have protective effects against cancer, cardiovascular disease, cataracts, macular diseases and immune disorders (Bendich, 1993).

Antioxidants are also important for industrial applications. For instance, proanthocyanidins, a naturally occurring antioxidant extracted from grape seeds are used as natural flavouring in industries in Japan (Nakamura *et al*., 2003). Besides, antioxidants are also added as food additives to add nutritional value and to extend the shelf life of food (Karawita *et al*., 2005).

2.4.2 Antioxidant Assays

Several methods and assays were developed to determine and quantify the presence of antioxidants. Colorimetric methods have been widely used to determine the presence of antioxidants particularly in food extract. The first example of antioxidants assay is the folin-ciocalteau assay. This assay is used primarily to detect the total polyphenol level in food. It is based on the chemical reduction of tungsten and molybdenum oxides, which are present in the reagent and work by measuring the amount of sample extract needed to inhibit the oxidation of the reagent (Waterhouse, 2002). On the other hand, vanillin-HCl assay is used for the general determination of condensed tannins in food. Condensed tannins or proanthocyanidins are flavonoids that consist of two or more flavan-3-ol such as catechin, epicatechin or gallocatechin (Sun *et al*., 1998). The vanillin used in the assay is protonated by acid solution, thereby producing weak electrophilic carbocation and react with the flavonoid ring. This dehydrated compound then gives a red coloured product.

Total antioxidant activity can be measured using the ferric reducing antioxidant power assay (FRAP). This method measures the presence of antioxidants through colour changes when ferric-tripyridyl-s-triazine complex is reduced to its ferrous coloured form (Hajimahmoodi *et al*., 2008). Reducing power assay on the other hand, is used to determine the reducing capability of the sample extract, in which the yellow colour of the test solution was reduced to either blue or green depending on the reducing power of the extract. The presence of antioxidants will reduce the ferricyanide (Fe^{3+}) complex to their ferrous form (Fe^{2+}) which can be observed by the formation of Perl's Prussian Blue and can be measured at 700 nm (Barros *et al*., 2007).

In addition to that, free-radical scavenging activity of an extract can also be determined using the DPPH assay. This assay uses 1,1-diphenyl-2-picryl hydrazyl (DPPH), as a free radicals generating compound to determine the radical scavenging activity of an extract. The DPPH free radicals are purple in colour and have a characteristic absorption at 517nm. The presence of radical-scavengers (or antioxidants) provides hydrogen atoms or electrons to the free radicals and thereby decreases the absorbance at 517nm (Blois, 1958). IC_{50} is often used to express the concentration of extract needed to reduce 50% of the free radicals and is inversely proportional to the scavenging activity of the sample extract. The drawback in the DPPH assay is that it reacts in a relatively slower speed, which can take up to a few hours to react. Besides, the presence of anthocyanins in the sample also leads to an under-estimation of the antioxidant activity in the extract due to colour interference (Arnao, 2000).

In the ABTS method, 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) is used as free radicals generators and the mechanism involved in the ABTS assay is the same as in the DPPH assay (Arnao, 2000). The oxygen radical absorbance capacity (ORAC) method is based on the inhibition of the peroxyl-radical induced oxidation initiated by thermal decomposition of azo-compounds, such as 2,2'-azobis (2-amidino propane) dihydrochloride (AAPH). The peroxyl radicals will react with the antioxidant compound and delay the degradation of fluoresin (Prior *et al*., 2003). The advantage of the ORAC method is that it can be used for both the hydrophilic and lipophilic antioxidants, thus providing better estimation of total antioxidant (Prior *et al*., 2003).

2.4.3 Previous Studies on Antioxidants from Plants

The consumption of antioxidant into the human body is through food intake or dietary supplement. Synthetic antioxidants such as β-carotene, vitamin A and vitamin E are widely sold in the market and they are found to increase the risk of mortality in human (Bjelakovic *et al*., 2007). The exact mechanism of action is unknown but may suggest the rigorous toxicity that they possess compared to natural antioxidants (Bjelakovic *et al*., 2007). Hence, there is a need to search for natural antioxidants sources to substitute the usage of synthetic one. Recent studies have been focusing on natural antioxidants in wine, fruits and vegetables due to their health benefits and commercial values.

Traditional plants with medicinal properties have also been studied widely for their antioxidant properties. For example, *Calpurnia aurea,* a small scrub which is widely distributed in South Africa has been used traditionally as treatment for amoebic dysentery and diarrhoea in animals, killing of head lice in humans, syphilis, wound scabies and different swelling (Fullas, 2001; Tadeg *et al*., 2005). Study conducted by Adedapo *et al*., (2008) strongly suggested that the high level of phenolics contents in this plants may contribute to some of its pharmacological effects. Besides that, *Melissa officinalis*, a medicinal plant used previously in folk medicine in Turkey and Iran and grown in the Mediterranean region was studied for their antioxidant properties (Sadraei *et al*., 2003). Their leaves were found to contain phenolic and flavonoids compounds that may contribute to their pharmaceutical and antioxidant properties (Chen *et al*., 2001).

Besides that, olive pulp extract was also studied as a potential source of natural antioxidants (Hajihmahmoodi *et al*., 2008). It was observed that the highest level of total polyphenol in the Iranian olive cultivar was found in the Mishen cultivar and

contained approximately 2.997±0.361 g GA/100g. In addition to that, sweet potato (*Ipomoea batatas*), an important crop especially in developing countries has been reported to be rich in minerals, dietary fibers, vitamins and antioxidants such as phenolic acids, anthocyanins, tocopherol and β-carotene (Woolfe, 1993). The phytochemicals found in sweet potato also displayed radical scavenging activity and several health promoting functions (Konczak-Islam *et al*., 2003).

In addition, the leaves and fruits of berry crops such as strawberry, raspberry and blackberry have also been reported to contain flavonoids, vitamin E, phenolics compounds, phenols (rosmaridiphenol, curcumin, butein), nitrogen compounds (alkaloids, chlorophyll derivatives), carotenoids and vitamin C which play important functions as antioxidants in these crops (Larson, 1988). Studies on mulberry fruits also found that these fruits are rich in antioxidant activity and were contributed by the presence of anthocyanin pigments (Bae and Suh, 2007). Hence, these fruits received wide attention due to their biological and pharmacological benefits contributed by their anthocyanins and flavonoids contents (Tsuda *et al*., 1999).

Aside from those stated above, antioxidant studies have also been conducted on a wide range of plants, vegetables and fruits. All these studies are important to provide essential information on the benefits of consuming the different food in our diet.

2.5 Introduction to Colouring Dyes

Dye can be defined as coloured, ionising and usually aromatic organic compound. Since they are aromatic compounds, their ability to exert colour will depends on their basic atomic structure of acryl ring (Fessedden and Fessedden, 1990). Human and some other animals have the ability to detect some wavelength of electromagnetic radiation (light) and thus have the ability to perceive different colours based on the electromagnetic wavelength. The presence of the ring in the compound holds the responsibility for the absorption of the different electromagnetic wavelengths (Fessedden and Fessedden, 1990).

Dye is used for the colouration of food, fabrics, and basically any objects that can be seen with eyes. It has been a long time since human discovered the usage of food colourings, which was believed to be dated back to the Ancient Romans (Straub, 2005). For instance, saffron and other spices have been used to colour food yellow. Besides, early humans also use minerals and ores such as azure (copper carbonate), gold leaf, and silver leaf as food colouring in the early discovery of minerals although some of these chemicals were poisonous if used improperly (Lansdown and Yule, 1984).

2.5.1 Synthetic Dye Versus Natural Dye

Synthetic dyes are prepared through chemical processes from various chemicals and man-made materials. The fascinations that colour brought to mankind cannot be underestimated as foods and items that have better appearance are fetching higher prices and are more preferred by consumers. Natural dyes from vegetables sources have been used since ancient time. However, since the emergence of synthetic dyes in 1856, the usage of natural dye has been limited (Murmann, 2000). The preference of using synthetic dyes in industries is due to synthetic dyes being cheaper, easier to use, more stable and more homogenous compared to natural dyes.

Currently, the usage of synthetic dye has created havoc to the environment such as water pollution in China, deemed one of the major hubs for producing dyed textiles for giant companies such as Gap, Target and Wal-Mart (Spencer, 2007). Although China has laws stipulating the processing of dye waste in water treatment plant before releasing them into the environment, the red coloured river downstream of these factories showed an estimated amount close to 22,000 tonnes of dye thus proving that laws and regulations were not sufficient to protect the environment if it is not implemented (Spencer, 2007).

Besides being harmful to the environment, the usage of synthetic dyes also resulted in negative effects on human health. The effects of six commonly used synthetic dyes such as tartrazine, quinine yellow, sunset yellow FCF, carmoisine, ponceau 4R and allura red (european codes E102, E104, E110, E122, E124 and E129) was included in the study conducted by Southhampton University (September 2007) (Tartrazine, 2010). The results from the study showed that banning the usage of the synthetic dyes could

prevent up to 30% of Attention Deficit Hyperactivity Disorder (ADHD) in children. It was also found that the worst dye could be compared to lead in petrol which lowers children IQ by 5 points (Tartrazine, 2010).

Besides that, the usage of the synthetic dyes such as malachite green as food colouring agent and food additive (Culp and Beland, 1996) has been reported to cause damage to the liver, spleen, kidney and heart, inflicts lesions on skin, eyes, lungs and bones as well as produces teratogenic effects in rats and mice (Werth and Boiteaux, 1967). In addition, tartrazine (FD&C Yellow nº5), one of the most widely used synthetic yellow dye in foods, drugs and cosmetic industries is a nitrous derivative and is known to cause allergic reactions such as asthma and urticaria (Moutinho *et al*., 2007). Tartrazine also remain as a focus of studies on mutagenesis and carcinogenesis due to its ability to transform into aromatic amine sulfanilic acid after metabolized by the gastrointestinal microflora (Moutinho *et al*., 2007).

Synthetic dyes used in industries were put into the limelight because the families of chemical compounds that make the good dyes are toxic to human. Each new synthetic dye developed is a brand new compound, and therefore their possible risks on human and environment are unknown. Many of these dyes such as Amaranth have entered the market, but were withdrawn later on after they were discovered to be carcinogenic (Tsuda *et al*., 2001). Hence, the usages of dangerous synthetic dyes formulated from toxic chemicals were banned by the European Union due to their adverse effects on human health (John, 2008). In order to reduce the usage of synthetic food dyes in human diet, the Delaney clause was established in 1958 under the Federal Food, Drug and Cosmetics Act 1958 which forbids the addition of any forms of chemicals into human food that can causes cancer (Weisburger, 1994).

Natural dyes on the other hand are obtained from nature such as fruits, vegetables and plants, which are regarded as renewable sources. Most of the natural colour sources are contributed by the presence of chromophores, pigments with ring structures that can absorb different light wavelengths and hence provide the various colours (Britton, 1983). The examples of natural dyes sources are marigold flowers, saffron, paprika, turmeric, annatto extract and beet root and their usages in industries are limited compared to that of synthetic dyes.

Therefore, consumers should be educated on the harmful effects of the synthetic dyes to allow them to make better choices in life. The usage of natural dyes is limited due to their higher cost of production and lesser stability. Besides, commercialisation of natural dyes would also require extensive research and studies to ensure consistency in their performance compared to synthetics dyes (Jothi, 2008).

2.5.2 Safety Issues Concerning Natural Dye from Plants

Natural dyes are commonly extracted from plant parts such as flowers, leaves, bark and roots. The major problem related to extraction from plant source is the remaining pesticides in the final product after the extraction process. The presence of pesticides residues in the consumer product especially food has been of major distress. United States Environmental Protection Agency (USEPA) had established procedures under the Miller Pesticide Amendment in 1954 to determine the safe amount of pesticides that can remain on raw agricultural products used as food. Pesticides toxicity is regarded as the adverse health effect observed following the interaction between the pesticides and the biological organisms (US-EPA, 1997). The current tolerance for pesticides residues in

food is 100 times below the level which observable health effects were detected in test animals. The tolerance values for the different pesticides can be accessed and viewed at the USEPA website (http://www.epa.gov/ pesticides/food/viewtols.htm).

Leaves are commonly treated with herbicides to prevent rodents, pest and fungus from attacking the plant. The usage of pesticides provides consumers with lower price and wider selection of agricultural products besides preventing disease outbreaks (from rodents and insects) in an area (US-EPA, 2012). The problem often associated with the usage of leaves as a source for extraction is the presence of insecticides and herbicides in the product of extraction. The name of the common pesticides found in agricultural products and the class they belong to are shown Table 2.1.

Table 2.1 Common Pesticides in Agricultural Products

Pesticide	Pesticide Class
Chlorpyrifos	Organophosphorus insecticide
Methyl parathion	Organophosphorus insecticide
Malathion	Organophosphorus insecticide
Atrazine	Herbicides
2, 4-D	Herbicides
Simazine	Herbicides
DDT	Organochlorine Insecticides
Endosulfan	Organochlorine Insecticides
Dieldrin	Organochlorine Insecticides

Exposure to commonly used organophosphorus insecticides such as chlorpyrifos, methyl parathion and malathion were found to decrease the antioxidant enzymes (catalase, superoxide dismutase and gluthione peroxidase) activities in rat (Ojha *et al*., 2011). Strict conditions have been imposed on the usage of chlorpyrifos, which focussed primarily on the health of infants and children (Overstreet, 2000). DDT leftover in the agriculture products are harmful to human such as increasing the risk of cancer (particularly liver cancer), damaging the reproductive system and temporarily damaging the nervous system (Longnecker *et al*., 1997).

Malathion was one of the earliest introduced organophosphate insecticides and was found to interfere with the normal function of the nervous system and cause breathing difficulty, vomiting, cramps, diarrhoea and possibly death in human. Besides that, Atrazine, the commonly used herbicide in agriculture was found to increase the chances of birth defects, breast cancer and causes problems to the reproductive health for both women and men. The presence of this herbicide in ocean was also found to be harmful to the aquatic organisms. The usage of endosulfan has been limited to agricultural and commercial used due to the over-stimulation activity on the nervous system in human who consumed it (Karatas *et al*., 2006). Dieldrin, categorised under the organochlorine insecticide was banned in the United States in 1974 because ingestion of this pesticide can cause convulsion and possibly death to human. Exposure to this chemical alone can cause headaches, dizziness, irritability and uncontrolled muscle movements.

Due to the negative effects observed from the usage of pesticides, in April 2012, the United States Environment Protection Agency has come out with the "Human Health Benchmarks for Pesticides" guideline for approximately 350 pesticides used in food crops. The benchmark level is set above the level which adverse health effects are observed from pesticides exposure (US-EPA, 2012). In addition, alternatives have been

suggested to overcome the dependence and negative effects of pesticides usage. This includes the usage of environmental friendly pesticides, rewarding farmers practicing organic farming method and establishing laws and regulations to control the usage of these chemicals. Therefore, the absence of pesticides residues in food products is the ultimate goal in industries and indeed a good sign for the potential consumer market.

In conclusion, more research has to be conducted to improve the extraction of natural dyes and to reduce their cost of production. Besides, the implementation and enforcement of laws and regulations on the safety of natural dye is also important to increase consumer confidence of natural dyes. It is hope that natural dyes will gain better acceptance compared to synthetic dyes in the future due to their eco-friendly property as well as their benefits to human health.

CHAPTER 3

Material Selection for Pigments Extraction:

General Screening of Pigments

3.1 INTRODUCTION

Recently, the extraction of carotenoids from *Ipomoea batatas* leaves has garnered economical importance as a natural source to complement the usage of synthetically derived chemical compounds. It is a safer choice and helps to utilise the *I. batatas* leaves, which were usually discarded upon harvesting (Wang *et al.*, 2010). Ishiguro and Yoshimoto (2007) were the first to discover the high level of lutein content in the leaves of *I. batatas* plants compared to the other plants leaves and this made the *I. batatas* leaves a suitable source of natural yellow dye. *Ipomoea batatas* or sweetpotato plants can be divided into many varieties and are cultivated worldwide for their storage roots. Although belonging to the same species, morphological study is important to allow better identification of the different varieties of the *Ipomoea batatas* leaves. This is also to ensure that the leaves, which are used as starting material can be correctly identified for extraction.

Material selection plays an important role in the extraction of dyes because different plant varieties will produce different level of pigments at different developmental stages. The production of pigments in plants are related to the physiological changes that occur in the plants and from factors such as soil condition, variety, developmental stages and growth conditions (Kimura and Roddriguez-Amaya, 2002).

Plants generally contain pigments or biochromes that are mainly used in the absorption of light. Pigments such as the chlorophylls are needed for plants to absorb sufficient amount of light for photosynthesis. During leaf development, the level of pigments in the leaves normally increases to provide energy through photosynthesis (Lefsrud *et al*., 2007). The main pigments found in leaves include chlorophyll, carotenoids, and anthocyanins. Chlorophylls are important because they served to absorb light during photosynthesis and for food production. Besides, carotenoids also play an important role in light absorption to complement the role of chlorophyll in plants.

The study of pigments such as chlorophylls and carotenoids in plant's leaf is well documented and usually involves the usage of solvents to extract the pigments from the plant. The extracted pigments can be analysed using a spectrophotometer or high performance liquid chromatography (HPLC). Both allow the pigment concentrations in leaf to be determined quantitatively. However, the usage of high performance liquid chromatography is often viewed as expensive and labour consuming and is not essential if the pigments composition is not the main objective. Besides the two methods mentioned above, other techniques such as thin layer chromatography, open column chromatography and liquid chromatography were also used to study pigments in plants.

The first objective of this chapter is to distinguish the different varieties of *I. batatas* through morphological variations and secondly, to determine and compare the levels of chlorophyll a, chlorophyll b, lutein and β-carotene pigments in the six different *I. batatas* leaves varieties at the different developmental stages using spectrophotometric method. The third objective is to determine the best storage temperature for the *I. batatas* leaves prior to extraction.

3.2 MATERIALS AND METHODS

3.2.1 Plant Materials

Ipomoea batatas leaves used in this experiment were collected from the *I. batatas* farm in Tanjung Sepat, Kuala Langat, Selangor (N02º 40.552' E101º 33.822'), which is located about an hour from the Post Harvest Biotechnology laboratory. A total of six different varieties of *I. batatas* leaves were used in this experiment, viz. *Ipomoea batatas* var. Batu Kelantan (BK), *Ipomoea batatas* var. Batu Biasa (BB), *Ipomoea batatas* var. Oren (Oren), *Ipomoea batatas* var. Indon (Indon), *Ipomoea batatas* var. Vitato (Vitato) and *Ipomoea batatas* var. Biru Putih (BP).

The leaves were tagged at the budding stage and were harvested according to the number of developmental days. All the varieties were grown under the same environment and soil conditions. Leaves were harvested randomly from the central row for all samples at the different developmental stages, namely young (1-3 days), immature (5-7 days), mature (9-12 days) and fully developed (15-18 days) stage. The formation of leaf bud at the petiole was calculated as day one of leaf development. The leaves were labelled and annotated with the date of collection and deposited at the Post Harvest Laboratory, University of Malaya.

3.2.2 Morphological Variation between the Different Varieties of *Ipomoea batatas* Leaves

Morphological study was performed on the six varieties of *I. batatas* leaves based on seven morphological characteristics namely: leaf width, length, shape, arrangement, margin, venation and surface type. Due to variation in leaf characteristics and practical constraints, maximum leaf width and length were chosen as the parameters. Maximum leaf width is the measurement at the widest point perpendicular to the midvein (Figure 3.1) (Williams and Martinson, 2003).

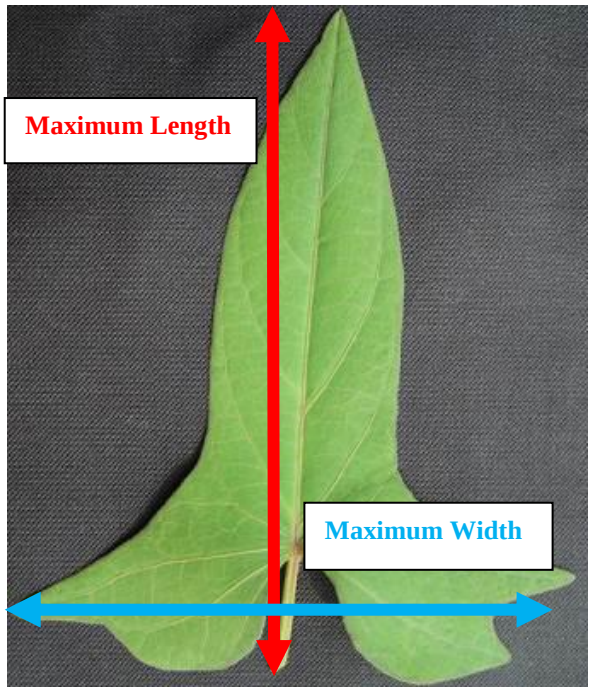


Fig 3.1 Method of measuring the maximum width and length for the leaves

3.2.3 Morphological Variation between the Different Varieties of *I. batatas* Storage Roots

The morphological differences between storage roots from the different varieties were determined and the four parameters studied were skin colour, flesh colour, shape and storage roots placement. A total of 50 samples (n=50) were used and the data was tabulated for comparison.

3.2.4 General Screening of Chlorophyll *a*, Chlorophyll *b*, Lutein and β-Carotene in *Ipomoea batatas* Leaves Using Spectrophotometric Method

One gram of finely ground fresh leaves was placed into a 100 ml conical flask and added with 50 ml of methanol (Merck). The mixture was shaken for 120 min under dim light condition at room temperature (25ºC). The mixture was then centrifuged for 15 min at 12000 rpm and 4ºC to avoid pigment degradation. The supernatant was collected for analysis.

The pigments in the methanol extract were determined using a spectrophotometer (MRC UV-200RS, Israel). Leaf extracts (30 µl) were added to 270 µl of solvent to give a total volume of 3 ml with 10x dilution from the actual concentration. A glass cuvette was used for spectrophotometric analysis. Absorbance readings were taken at 652 nm and 665 nm. The amount of pigments in the leaves was calculated based on the equation provided by Lichtenthaler and Buschmann (2001). The experiment was performed in triplicates and the formulas used for the calculation of these pigments are as shown below:

(c_a and c_b represent chlorophyll *a* and chlorophyll *b* in the leaves extract respectively.)

c_a (µg/ml) = 16.72 A_{665} – 9.16 A_{652}

c_b (µg/ml) = 34.09 A_{652} – 15.28 A_{665}

The levels of the lutein and β-carotene pigments in the *I. batatas* leaves were determined using spectrophotometric method as described previously by Bulda *et al*. (2008) with some modifications. The liquid nitrogen frozen sample (0.5 g) was ground in a mortar and pestle in 2 ml of 1:1 mixture of petroleum ether (PE) and tetrahydrofuran (THF). Petroleum ether (3 ml) was added and the mixture was ground until homogenous. The mixture was filtered through Whatman No. 1 filter paper. Saponification was then performed to remove chlorophylls and lipids from the extract. Three microliters of freshly prepared KOH (1 g/ml) was added to 10 ml of the extract. The mixture was incubated for 5 min at 45ºC. The solution was cooled on ice and the sample was left to settle. The uppermost coloured fraction was subjected to absorbance at 480, 495, 645 and 655 nm in a spectrophotometer. The corrected absorbance readings at 480 nm and 495 nm were then calculated by subtracting the absorption coefficient of the chlorophylls *a* and *b* to remove the possible interference by the chlorophylls (at 645 and 655 nm).

$A_{480} = A°_{480} – 0.566\ A_{645} + 0.121\ A_{655}$

$A_{495} = A°_{495} – 0.112\ A_{645} – 0.0036\ A_{655}$

(A is the corrected absorbance and A° is the actual absorbance reading)

The concentration of lutein (C_{lut}) and β-carotene ($C_{\beta\text{-car}}$) level was calculated using the formula below and plotted into a graph.

C_{lut} (g/L) = 11.51 A_{480} – 20.61 A_{495}

$C_{\beta\text{-car}}$ (g/L) = 17.16 A_{495} – 3.96 A_{480}

3.2.5 Statistical Analysis of Data

Pigments analyses were performed in triplicates and the mean data were tabulated. Statistical analyses were performed using one-way ANOVA and Tukey's test using SPSS software (SPSS 19, IBM).

3.2.6 Sample Handling and Storage

I. batatas leaves harvested from the farm were brought back to the laboratory, washed and left to dry before pigments analyses were conducted. The remaining leaves were ground in liquid nitrogen and stored at -80ºC until analysis. The storage temperatures between harvesting and grinding process can influence the pigments concentrations in the leaves samples. The harvested leaves were subjected to three different storage temperatures: room temperature (25°C), 15ºC and 4ºC to determine the best storage temperature and maximum storage length for the leaves.

The concentrations of lutein and β-carotene in the leaves were determined spectrophotometrically (as described in section 3.2.4) on the day of harvest (day 0), day 2, day 4 and day 6. The means of the lutein and β-carotene concentrations extracted at different storage durations were calculated and presented in graph. All the analyses were done in triplicates and means were accompanied by standard errors bar.

3.3 RESULTS

3.3.1 Morphological Variation between the Different Varieties of *Ipomoea batatas* Leaves

Leaf morphology was used as an important parameter to distinguish the different varieties of *I. batatas* leaves used in this study. Figure 3.2 shows photographs of the different varieties of the *I. batatas* leaves collected.

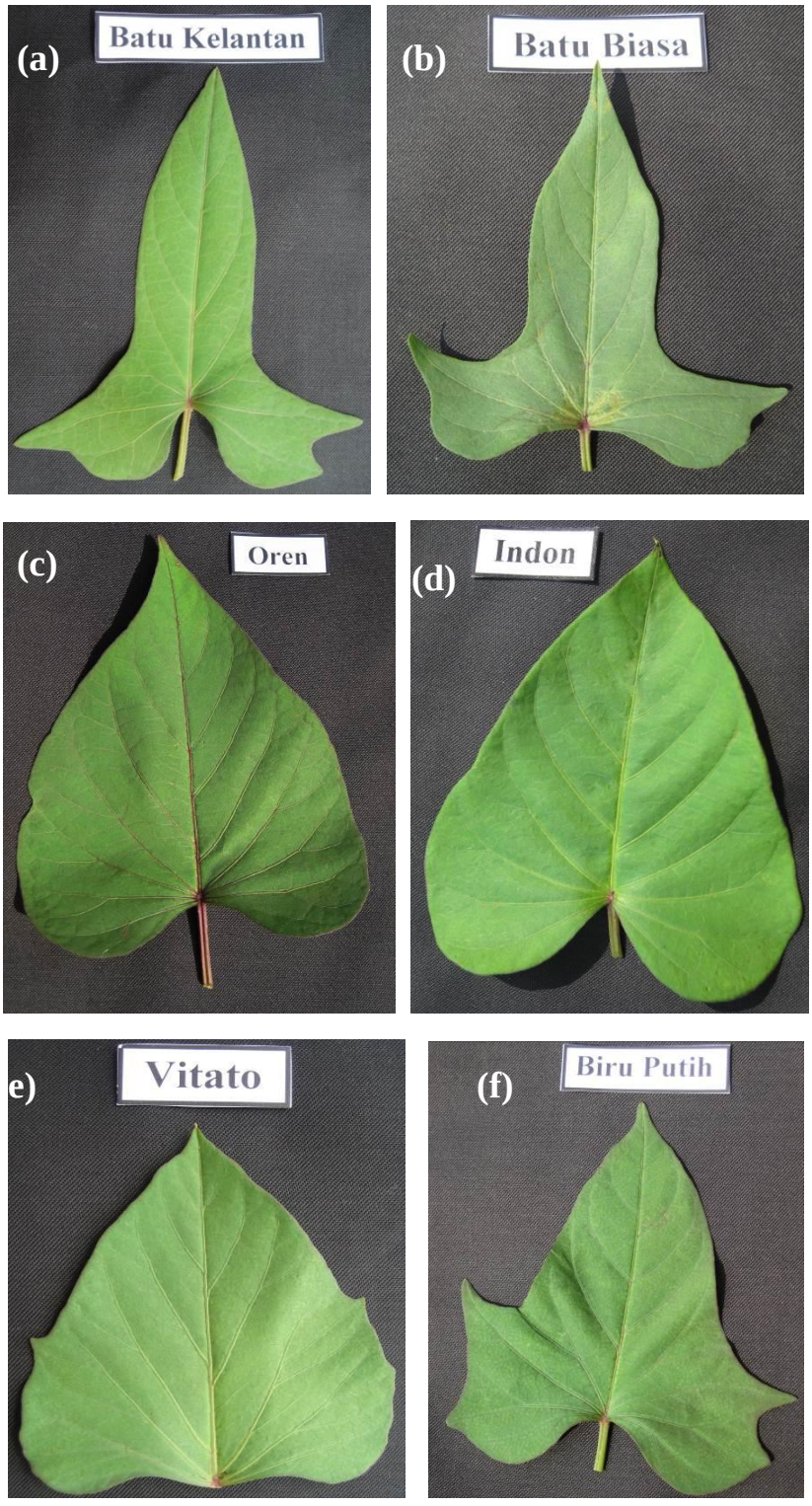


Fig 3.2 Photographs of different varieties of *Ipomoea batatas* leaves

The different leaves varieties studied: (a) *Ipomoea batatas* var. Batu Kelantan, (b) *Ipomoea batatas* var. Batu Biasa, (c) *Ipomoea batatas* var. Oren, (d) *Ipomoea batatas* var. Indon, (e) *Ipomoea batatas* var.Vitato, (f) *Ipomoea batatas* var. Biru Putih

Table 3.1 showed the leaf morphologies for the different varieties of *I. batatas* plants. Both the Batu Kelantan (BK) and Batu Biasa (BB) varieties have hastate shaped leaves while the Oren, Indon and Vitato (Vit) varieties have cordate shaped leaves. The Biru Putih (BP) variety however has cordate shaped young leaves and hastate shape for the older leaves.

Leaf arrangement refers to the position of leaves on the stem of the plant and all the *I. batatas* varieties in this study had alternate arrangement for their leaves. Besides, all varieties studied also showed similarities in their entire margin and arcuate venation. Margin is the term used to describe the side pattern of the leaf while the venation pattern of the leaves refers to the arrangement of veins on the leaves surface. In addition to that, the leaves were also distinguished based on the pattern of their apex and their base; apex refers to the pointed tips of the leaves while the base connects the leaves to the petiole. All the varieties has caudate shaped apex except for the Indon variety, with broadly arcuate apex. On the other hand, the BK and BB varieties both have hastate shaped base compared to the others with cordate shaped leaves.

Leaf surface can also be used to distinguish different leaf varieties. The BK and BB varieties has waxy surface for their young leaves, while the BP variety has young leaves with non-waxy and rugose surface. The Oren, Indon and Vitato varieties have waxy and rugose young leaves compared to glabrous mature leaves surface. Table 3.1 summarised the differences in leaf morphology for the six *Ipomoea batatas* varieties.

The variations in the leaf morphologies included parameters such as leaf shape, arrangement, margin, venation, apex, base and surface type for the six different varieties of *Ipomoea batatas* leaves studied (n=50).

Table 3.1 Leaf Morphology for Six Varieties of *Ipomoea batatas*

Variety	BK	BB	Oren	Indon	Vit	BP
Shape	Hastate	Hastate	Cordate	Cordate	Cordate	Cordate (Y) & Hastate (O)
Arrangement	Alternate	Alternate	Alternate	Alternate	Alternate	Alternate
Margin	Entire	Entire	Entire	Entire	Entire	Entire
Venation	Arcuate	Arcuate	Arcuate	Arcuate	Arcuate	Arcuate
Apex	Caudate	Caudate	Caudate	Broadly acute	Caudate	Caudate
Base	Hastate	Hastate	Cordate	Cordate	Cordate	Cordate
Surface :						
(a) Young	Waxy	Waxy	Waxy, Rugose	Waxy, Rugose	Waxy, Rugose	Non-waxy, Rugose
(b) Mature	Glabrous	Glabrous	Glabrous	Glabrous	Glabrous	Glabrous

The different development stages of the leaves can be identified from the leaf length and width. The widths of the leaves were measured at day 1-3, day 5-7, day 9-12 and day 15-18 of the leaf developmental stage and were tabulated in Table 3.2. The first day of leaf development implies the formation of the leaf bud at the petiole.

Table 3.2 Mean Width of Different Varieties of *Ipomoea batatas* Leaves at Different Development Stages

Variety	Width (cm)			
	Day 1-3	Day 5-7	Day 9-12	Day 15-18
BK	2.2 ± 0.3^{ac}	5.5 ± 0.4^{ab}	9.6 ± 0.4^{ad}	12.5 ± 1.8^{ad}
BB	2.3 ± 0.3^{b}	6.5 ± 0.6^{ab}	9.4 ± 0.7^{b}	13.2 ± 0.7^{bd}
Oren	1.7 ± 0.4^{abc}	4.9 ± 0.5^{ab}	9.4 ± 0.6^{c}	11.7 ± 0.9^{cd}
Indon	2.8 ± 0.5^{abc}	5.8 ± 0.7^{b}	9.4 ± 1.0^{ade}	12.8 ± 0.6^{abcde}
Vitato	2.5 ± 0.5^{ab}	6.5 ± 0.7^{b}	10.0 ± 0.9^{d}	14.1 ± 0.9^{abce}
BP	3.1 ± 0.7^{abc}	6.4 ± 0.5^{a}	9.4 ± 0.7^{cde}	12.4 ± 0.5^{de}

The similar letters beside the mean width indicate the statistically significant differences between the different leaves varieties at the $P<0.05$ level in Tukey's HSD test (n=20).

Besides width, the length of the leaves was also studied as parameter to identify the different stages of the leaf development. The length of the leaf was measured from the base to the tip of the leaf. The Batu Biasa variety has larger leaf length compared to the other varieties at the earlier stage of development. The Oren variety has smaller leaf length compared to the others throughout the developmental stages.

Table 3.3 Mean Length of Different Varieties of *Ipomoea batatas* Leaves at Different Development Stages

Variety	Length (cm)			
	Day 1-3	Day 5-7	Day 9-12	Day 15-18
BK	2.8 ± 0.6^{ab}	6.4 ± 0.7^{a}	9.3 ± 0.5^{a}	13.1 ± 0.6^{ad}
BB	3.4 ± 0.7^{abcde}	6.5 ± 0.6^{b}	9.4 ± 0.7^{b}	13.2 ± 0.7^{bd}
Oren	2.4 ± 0.7^{bc}	6.4 ± 0.6^{c}	8.5 ± 0.7^{abde}	10.9 ± 0.6^{abcd}
Indon	2.8 ± 0.5^{bd}	5.8 ± 0.7^{abcd}	9.4 ± 1.0^{c}	12.8 ± 0.6^{c}
Vitato	2.5 ± 0.5^{be}	6.5 ± 0.7^{d}	9.9 ± 0.9^{d}	14.1 ± 0.9^{abcd}
BP	3.1 ± 0.7^{c}	6.4 ± 0.5^{e}	9.4 ± 0.7^{e}	12.4 ± 0.5^{abd}

The similar letters beside the mean length indicate the statistically significant differences between the different leaves varieties at the $P<0.05$ level in Tukey's HSD test (n=20).

Besides leaves, the differences in the storage root morphology were also used to determine the different varieties of the *Ipomoea batatas*. Photographs of the different varieties of *Ipomoea batatas* storage roots are shown in Figure 3.3 and Table 3.4 shows the differences in the storage root morphology for the different varieties of the *Ipomoea batatas*. A total of four root parameters were taken into consideration, which included root skin colour, flesh colour, shape and storage root placement.

Both the Batu Kelantan and Batu Biasa varieties have smaller light brown skin and yellow flesh. However they can be distinguished by their shape of the storage roots. While the Batu Kelantan's storage roots are round in shape, the Batu Biasa had narrow and small storage roots. The Oren variety was different from the others because this variety has round shape purple coloured skin with orange coloured flesh storage roots. The Indon variety has purple coloured flesh has brown skin colour and was slightly longish in shape. The Vitato variety has light brown skin colour and orange flesh whereas the Biru Putih variety has cream coloured skin and variegated pink flesh colour. The Biru Putih variety is different from the others in their storage roots placement because their storage roots are located far from their main root compared to the other varieties.

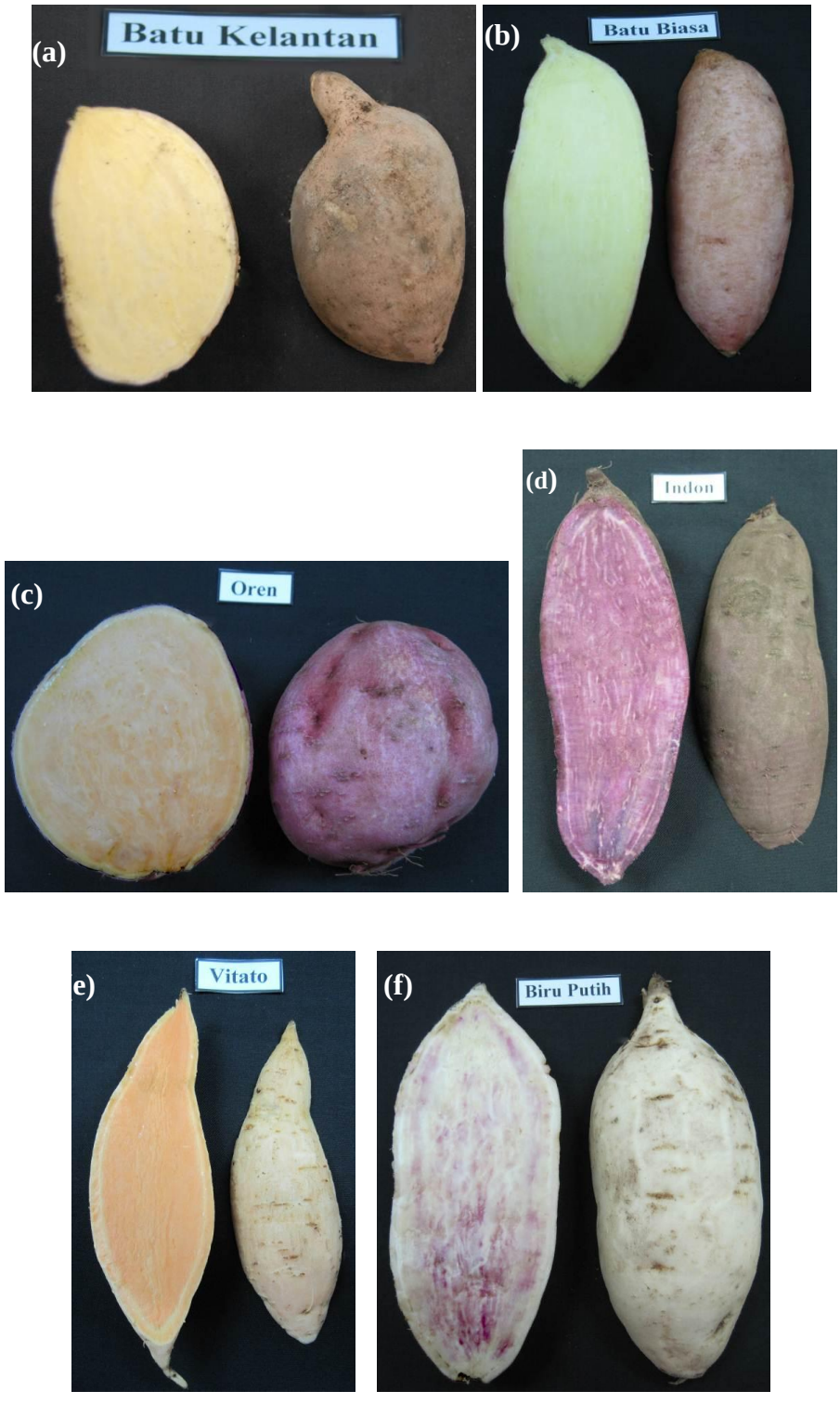


Fig 3.3 Photographs of storage roots for the different varieties of *I. batatas*

The storage roots of the different varieties studied were: (a) *Ipomoea batatas* var. Batu Kelantan, (b) *Ipomoea batatas* var. Batu Biasa, (c) *Ipomoea batatas* var. Oren, (d) *Ipomoea batatas* var. Indon, (e) *Ipomoea batatas* var.Vitato, (f) *Ipomoea batatas* var. Biru Putih

Table 3.4 Storage Root Morphology for the Different *I. batatas* Varieties (n=50)

Variety	Skin colour	Flesh colour	Shape	Storage Roots placement
Batu Kelantan	Cream	Yellow	Round	Close to main root
Batu Biasa	Light brown	Cream	Narrow and small	Close to main root
Oren	Purple	Orange	Round	Close to main root
Indon	Brown	Purple	Longish	Close to main root
Vitato	Light brown	Orange	Longish	Close to main root
Biru Putih	Cream	Variegated Pink	Longish	Far from main root

3.3.2 General Screening of Chlorophyll *a*, Chlorophyll *b*, Lutein and β-Carotene in *Ipomoea batatas* Leaves using Spectrophotometric Method

The level of total chlorophyll (sum of both chlorophyll *a* and chlorophyll *b*) were determined spectrophotometrically and the results are shown in Figure 3.4. Leaves were harvested at different developmental stages and the total chlorophyll was determined. The Indon variety showed overall lower chlorophyll level compared to the other varieties, while the Oren variety exhibited the highest chlorophyll level from day 9 onwards compared to the others. The total chlorophyll level in all the varieties rose sharply in the early stages of development and the level of total chlorophyll in the Oren, BK, Vitato and Indon varieties increased until day 12 before the level declined as senescence occurred towards the end of leaf development. Both the BP and BB varieties however showed a similar pattern of decrement in the total chlorophyll level after day 7.

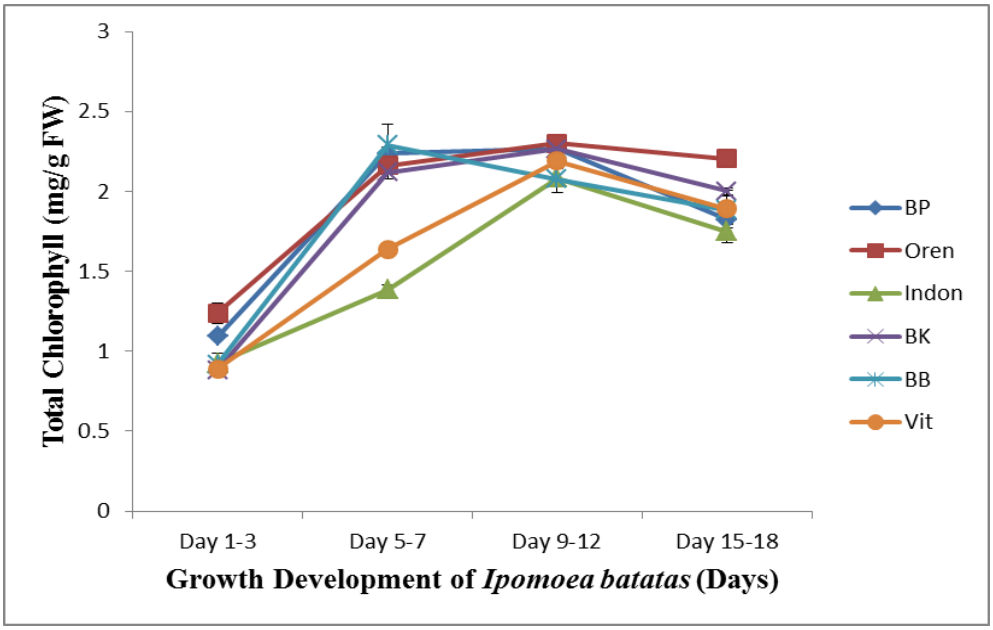


Fig 3.4 Total chlorophyll levels in the different growth stage of *I. batatas* leaves

The carotenoids pigments in leaves contributed to the yellow colour in nature. The concentration of lutein was measured at the different stages of leaf development and the data are tabulated in Figure 3.5. The lutein concentrations in the leaves were plotted against the days of leaf development for the different varieties of *Ipomoea batatas*. As can be seen from Figure 3.5, the Indon variety showed overall the lowest lutein concentration at all stages of leaf growth and development. All the *I. batatas* varieties showed a similar pattern in lutein level throughout the different stages of growth starting with a large increment at the early growth stage, followed by an almost stagnant level and a drop at the later stages of growth development.

Besides lutein, β-carotene pigments also contribute significantly to the carotenoids composition in the leaves. From Figure 3.6, the β-carotene level was found to be highest in the Oren variety while the Indon variety exhibited the lowest level of β-carotene. The Oren, BK and BP varieties showed continuous increment in their β-carotene level with the largest increment observed at the earlier stage of growth development. However, the concentrations of β-carotene in the BB, Vitato and Indon varieties declined from day 12 onwards.

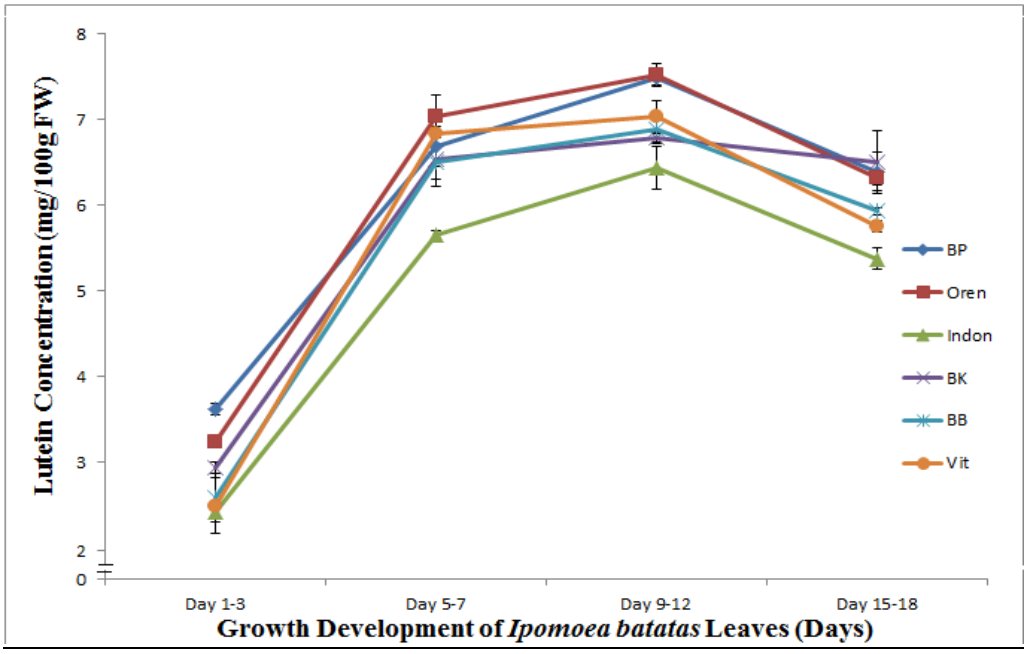


Fig 3.5 Lutein concentration in the different growth stage of *I. batatas* leaves

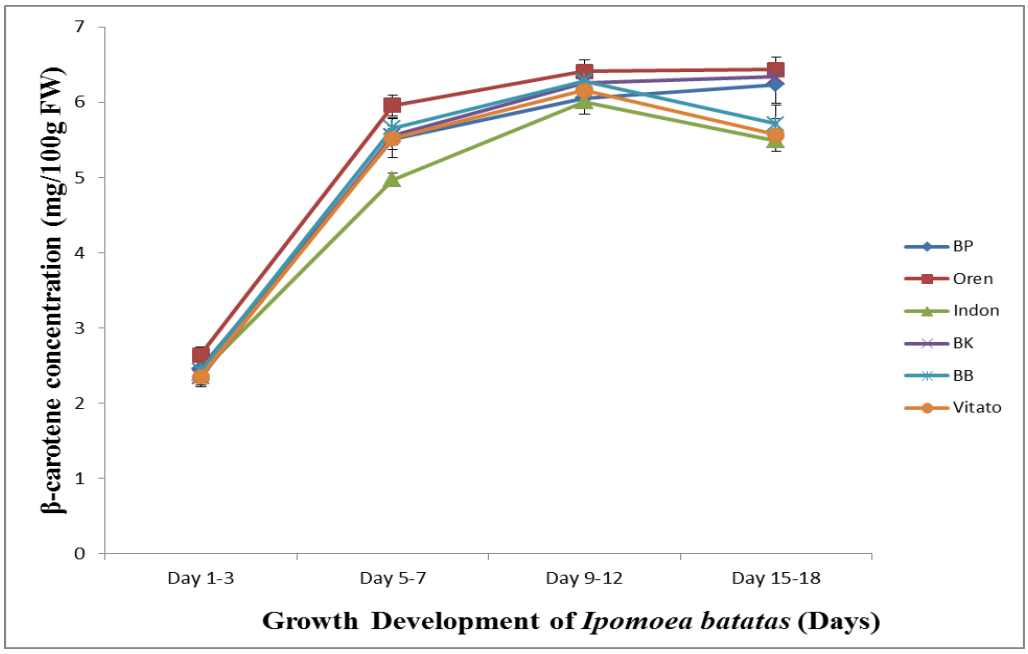


Fig 3.6 β-carotene concentration in different growth stage of *I. batatas* leaves

.

The relationship between the level of chlorophyll and the two major carotenoids (lutein and β-carotene) were studied and is shown in Figure 3.7. The highest correlation between total chlorophyll and lutein were demonstrated in the BK variety whereas the highest correlation for total chlorophyll and β-carotene were present in the Oren variety. Overall, all the varieties showed high correlation between the level of total chlorophyll and carotenoids in the leaves, i.e high level of chlorophyll indicates high level of carotenoids in the leaves. Therefore, the selection of intensely green coloured leaves during harvesting as the starting material will ensure optimum carotenoids extraction from these leaves.

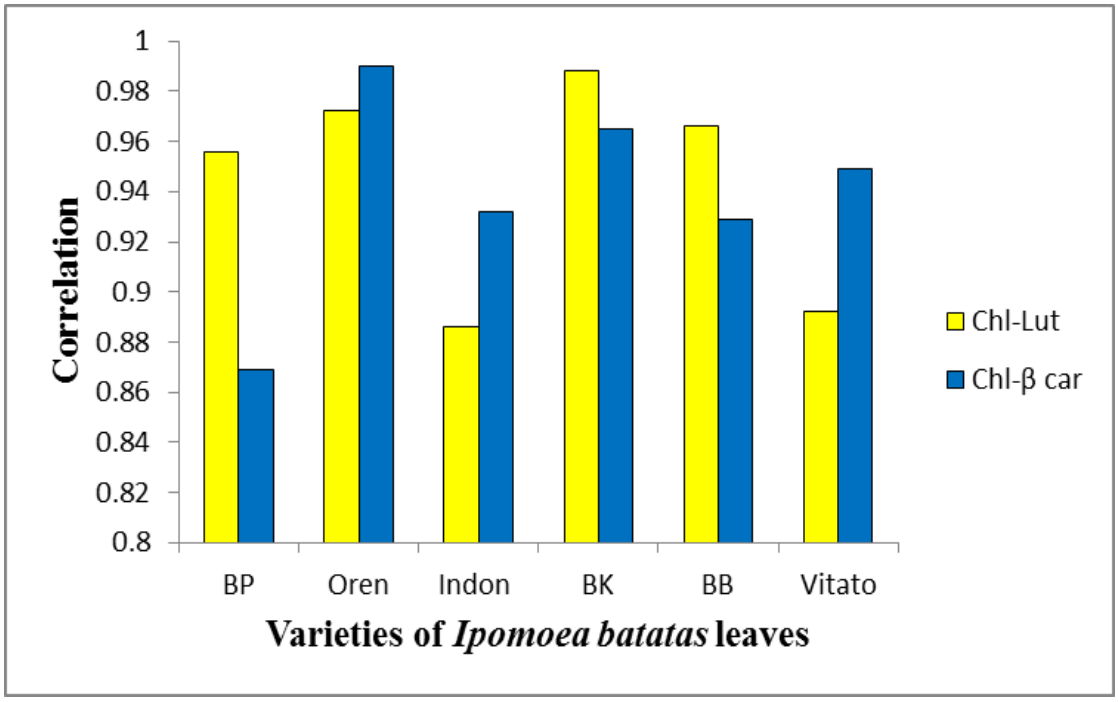


Fig 3.7 Correlation between total chlorophyll and lutein and total chlorophyll and β-carotene

3.3.3 Sample Handling and Storage

Harvested leaves samples (approximately 5 days and above) were kept under different storage temperatures upon harvesting prior to extraction. The leaves were kept at room temperature (25ºC), 15°C and 4ºC. The level of β-carotene and lutein in the leaves were determined using the spectrophotometric method as described in Section 3.2.4.

Figure 3.8 showed the percentage of lutein level in the different varieties of *Ipomoea batatas* leaves when stored at 4°C. It was observed that for all the varieties studied the lutein level in the leaves fell dramatically from day 2 onwards. Black spots and necrosis were observed on the leaves of the Vitato, BK and BP varieties on day 6 and therefore the leaves could not be used for extraction. The Oren variety had the highest percentage of lutein concentration left after 6 days of storage compared to the other leaves varieties. All the analyses were done in triplicates and results were shown in means accompanied by standard errors bar.

Figure 3.9 shows the percentage of β-carotene in the six different varieties of *Ipomoea batatas* leaves kept at 4°C during six days of storage. A slight decrease in the β-carotene concentration was observed initially until day two. A larger decrease followed from day 2 onwards and at day 6, necrosis was observed on the margin of most of the leaves. The Oren variety showed highest durability by retaining the highest percentage of β-carotene in its leaves compared to the other varieties.

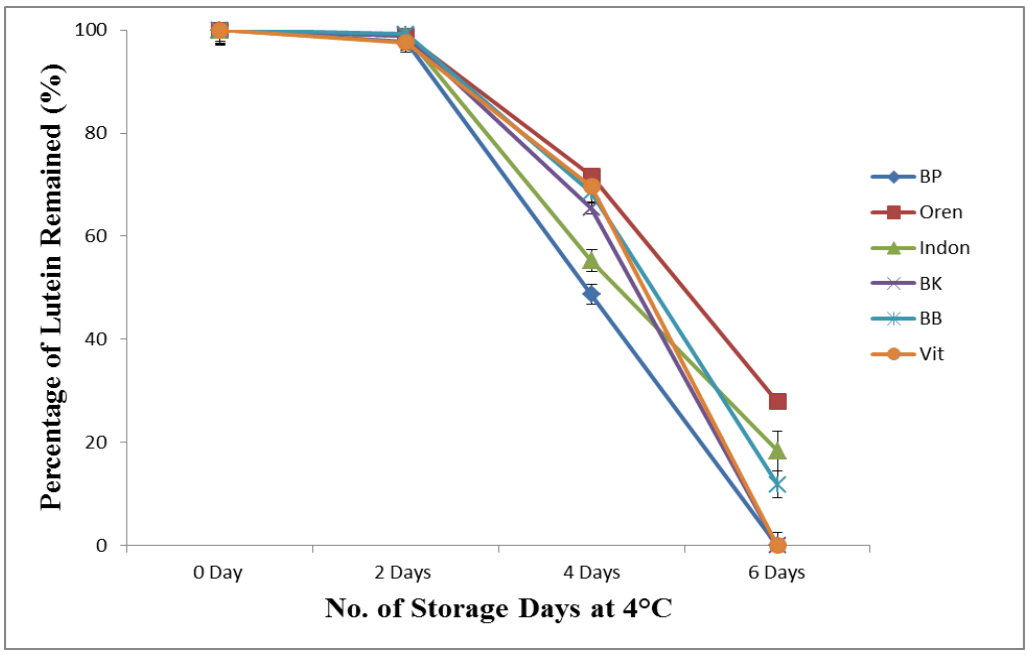


Fig 3.8 Percentage of remaining lutein in the *I. batatas* leaves kept at 4°C

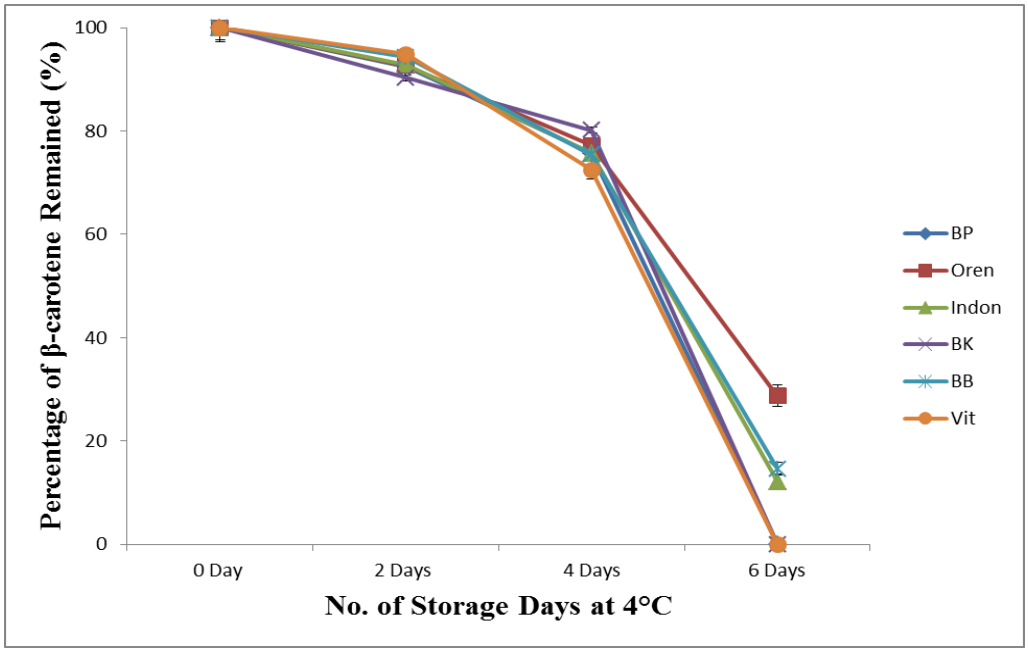


Fig 3.9 Percentage of remaining β-carotene in *I. batatas* leaves kept at 4°C

Figure 3.10 showed the percentage of lutein in the different varieties of *Ipomoea batatas* leaves when stored at 15ºC. A slight decrease was observed for the percentage of lutein from the day of harvesting to the second day of storage. From day 2 onwards, large decrease was observed on the percentage of lutein in the leaves with only approximately 20% pigments remaining at the sixth day of storage. At day 6, the Oren variety retained the highest percentage of lutein in the leaves. It was observed that the necrosis suffered by the leaves at day 6 were less severe compared to leaves stored in 4ºC.

Figure 3.11 shows the percentage of β-carotene in the different varieties of leaf samples stored at 15ºC. The percentage of β-carotene dropped slightly from the day 0 to day 2 and decrease steeply from day 4 to day 6. The Vitato leaves exhibited the lowest percentage of β-carotene at the sixth day of storage with approximately 20% of β-carotene pigment. Necrosis was observed on the side margin of the Vitato leaves on the 6^{th} day of storage.

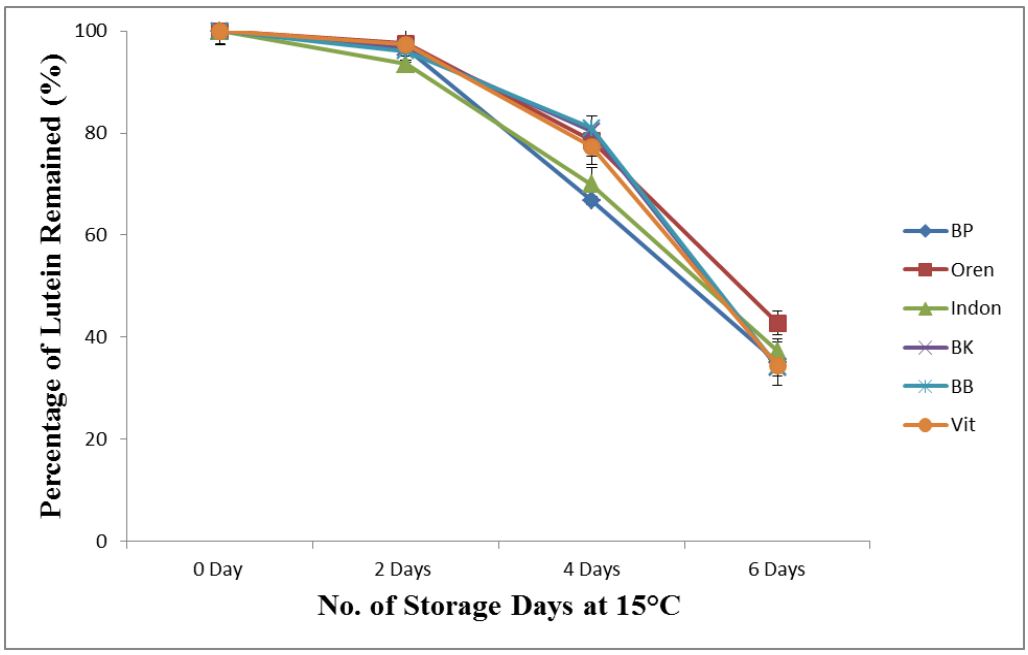


Fig 3.10 Percentage of remaining lutein in *I. batatas* leaves kept at 15ºC

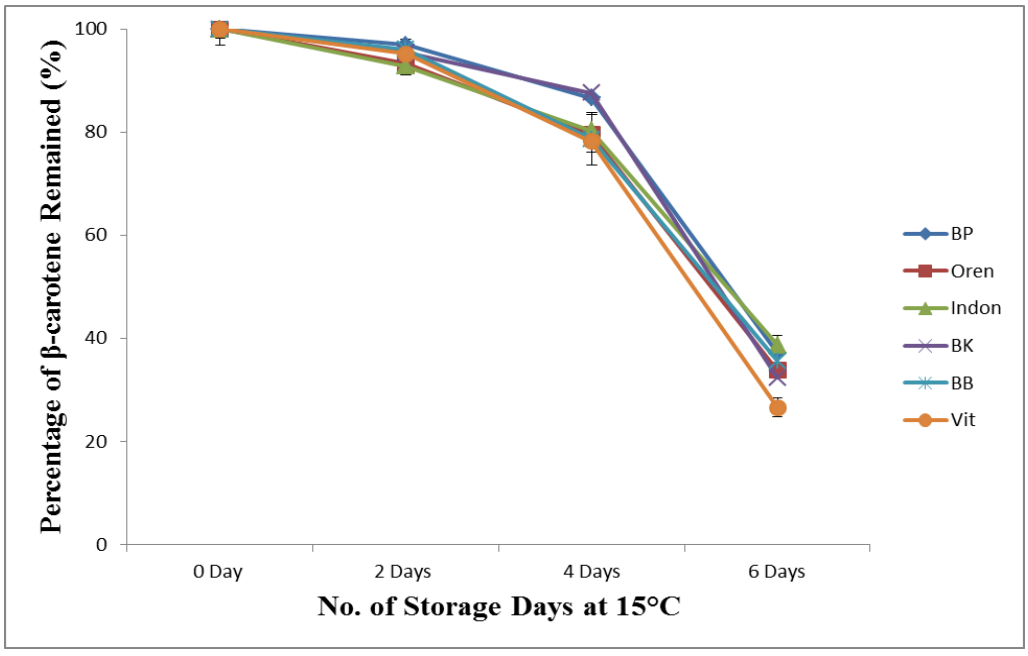


Fig 3.11 Percentage of remaining β-carotene in *I. batatas* leaves kept at 15ºC

Figure 3.12 showed the percentage of lutein in the different varieties of *I. batatas* leaves when stored at room temperature (25ºC). Continuous decrement was observed in the lutein level from the day of harvest throughout the storage period. At the fourth day of storage, only the Oren and Vitato leaves can be used for analysis as leaves from other varieties suffered from severe necrosis and black spots formation. However, at day 6, all the leaves were severely damaged and cannot be used for extraction. Hence, the harvested leaves were not suitable to be kept under room temperature in order to maintain the quality and quantity of the pigment in the leaves samples.

Figure 3.13 shows the percentage of β-carotene remained in the different varieties of leaf samples stored at room temperature (25ºC). The percentage of β-carotene concentration dropped sharply from the day of harvest up to day 4 and only the Oren and Vitato leaves can be used for extraction. The leaves of other varieties suffered from necrosis starting from the 4^{th} day of storage and therefore cannot be used for extraction.

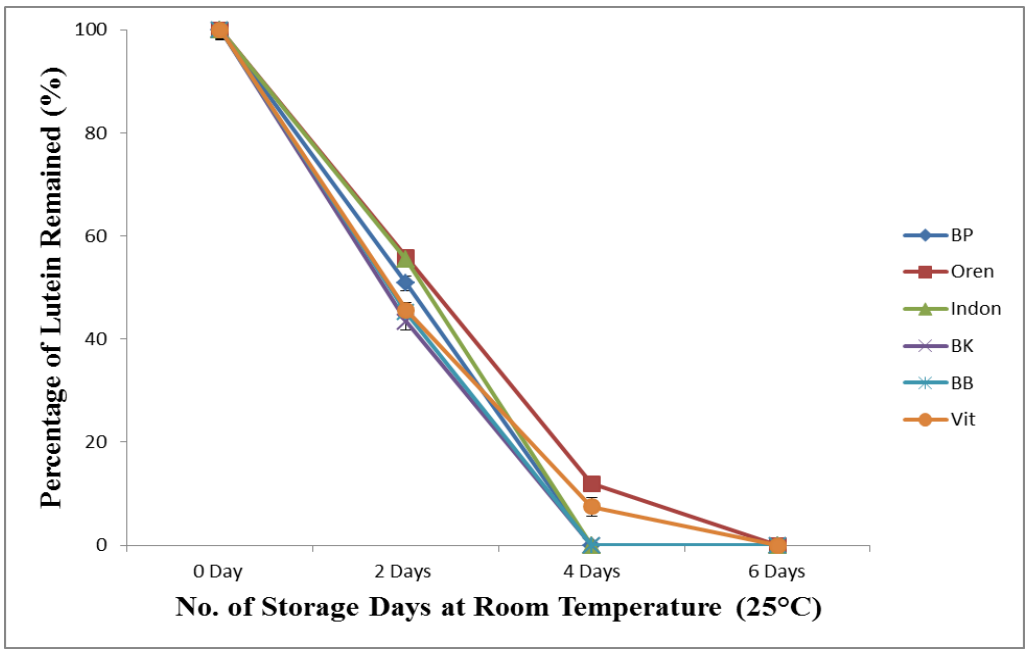


Fig 3.12 Percentage of remaining lutein in *I. batatas* leaves kept at room temperature (25°C)

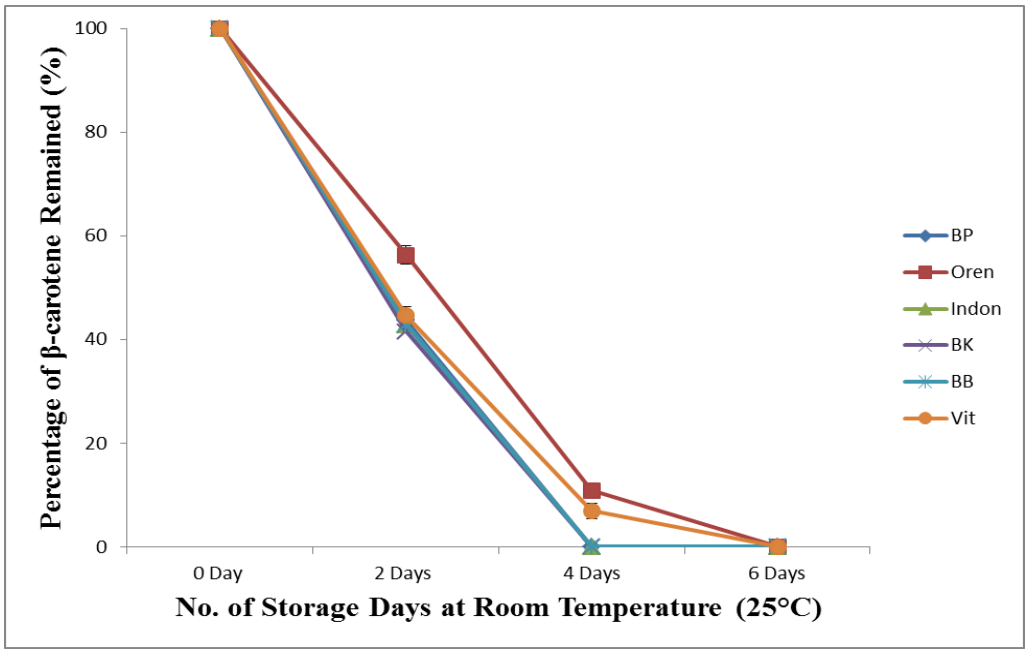


Fig 3.13 Percentage of remaining β-carotene in *I. batatas* leaves kept at room temperature (25°C)

3.4 DISCUSSION

Ipomoea batatas can be divided into various varieties such as Beauregard, Carolina Bunch, Centennial, Georgia Jet, Kotobuki (Japanese) and Satsuma (Japanese) (Bailey, 2011). Like in the other parts of the world, the *I. batatas* varieties used in this study were also known based on their demographic area. For instance, the Satsuma variety was name as such since it was planted in Satsuma (Kagoshima) region in Japan (Bailey, 2011). The six varieties of *I. batatas* used in this study are common varieties planted in Malaysia.

Due to the high numbers of varieties in *I. batatas*, it is often a tedious task to distinguish among them. While current method uses the variations of the storage roots morphology to distinguish between the different varieties, leaves morphology were also observed to vary in the different varieties. It is important to identify and distinguish between the different varieties of *I. batatas* so that subsequent material selection for extraction can be done in an accurate manner. Physical morphological variations are selected because they are cheap, fast and easy methods to distinguish among the different varieties.

While it has been observed that variation in plant size reflects primarily on the different environmental conditions, variations in leaf shape are often more inheritable and independent of the environment and therefore a good parameter to distinguish between the different varieties (Dickinson *et al*., 1987). It has been documented that the shape of the leaf is a response of the plant to long-term ecological and evolutionary history. Besides, limiting factors from the environment may also modify the finished form and shape of a tree's leaves (The Pennsylvania State University, 2009). The presence of complex edges and lobes in larger leaves enable them to disperse absorbed heat very rapidly. Besides, leaves' surface area will also affect the transpiration rate in the plant,

i.e larger surface area reflects higher transpiration rate in the plant (The Pennsylvania State University, 2009). In this study, all the *Ipomoea batatas* varieties have entire margin in their leaves with no presence of lobes and edges and thus, it can be deduced that the plant is well equip with good cooling system to help transmit heat from the plant.

Besides leaves, *I. batatas* storage roots' are also used to supplement the identification process between the different varieties. The important parameters taken into accounts are the storage roots' skin colour, flesh colour, shape and the storage roots placement. While leaves morphologies are important to visually distinguish between the different varieties of *I. batatas*, it is also important to differentiate the developmental stages of these leaves. This is because leaves at different developmental stages have different pigments levels, which may influence the choice of leaves material used in extraction. The developmental stages of the leaves were distinguished based on the number of development days for the leaves. Leaves development in this study were determined based on their width and length and therefore will ease the leaves selection process for extraction. The formation and presence of the different pigments in the *I. batatas* storage roots are contributed by the different biochemical pathways that occurred in the plant. For instance, the presence of the anthocyanin pigments in the storage roots are contributed by the flavonoid biosynthesis pathway whereas the carotenoids pigments are produced by the carotenoid biosynthesis pathway (Tanaka, 2008).

The selection of a suitable extraction source is the key step for the subsequent extraction process. However, the amount of carotenoids in green leafy vegetables are subjected to natural variation, variety or cultivar, climate, stage of maturity and postharvest handling practices and these factors may account for part of the divergence for the same plants (Chen and Chen, 1992; Kimura and Roddriguez-Amaya, 2002). Pigments accumulation

studies have been conducted in plants such as kale, clover and tomato leaves (Yoo *et al*., 2003). However, no study has been conducted for the pigments levels in the different varieties of the *Ipomoea batatas* leaves.

In the extraction of natural yellow dye, the levels of lutein and β-carotene are particularly important to select for the most suitable source for extraction. Total chlorophylls pigments found in leaves can be divided into chlorophyll *a* and chlorophyll *b*. Chlorophyll *a* is the major pigment in plants that absorb light of all wavelengths except green and therefore gives leaf its green colour. Besides, leaves also contain other accessory pigments such as chlorophyll *b* and carotenoids (including lutein and β-carotene) which absorb energy not absorbed by chlorophyll a. The changes observed in the concentrations of these pigments occur as plants grow and reach maturity due to the physiological changes that occur in the plants during the growing cycle. Deterioration of chlorophyll levels usually represents the senescence process that took place in leaf, resulted in the appearance of yellow, red or orange leaves (Barry *et al*., 1992).

Although the level of chlorophyll *a* and chlorophyll *b* (total chlorophyll) in leaves does not influence the extraction of the natural yellow dye as they are removed during the saponification process, these pigments allows the study of the physiological state of the plant during the different stages of development. The total chlorophyll content extracted from the *I. batatas* leaves were compared with other plants using the same protocols adopted from Lichtenthaler and Buschmann (2001). Beech (*Fagus Sylvatica*) showed total chlorophylls of 2.516 mg/g FW in the sun leaves and 4.204 mg/g FW in shade leaves whereas hornbeam (*Carpinus betulus*) showed 3.260 mg/g FW in the sun leaves and 6.670 mg/g FW for the shade leaves. Besides that, the dark green sun leaves of the poplar (*Populus nigra*) plant exhibited 3.212 mg/g FW of total chlorophylls content compared to 4.346 mg/g FW in the dark green shade leaves (Lichtenthaler and

Buschmann, 2001). The total chlorophyll extracted from the *I. batatas* leaves amounted up to 2.264 mg/g FW at the peak concentration and thus can be categorised as a sun leaves.

Generally, sun leaves contained thicker cell walls (thicker palisade layer) and lower water content (50% to 65% of fresh weight) compared to shade leaves (68% to 85% of fresh weight). Besides, sun leaves also received higher amount of light compared to shade leaves and thus have higher photosynthetic activity (Björkman, 1973). The higher photosynthetic activity will increase the numbers of carotenoids pigments in the leaves which are beneficial for the natural yellow dye extraction. Study conducted by Lichtenthaler in 1987 suggested that the percentage of carotenoids in green leaves vary in ranges for lutein (40-60%), β-carotene (25-40%), violaxanthin (10-20%) and neoxanthin (5-13%) pigments. In addition, lutein is also known to contribute the highest portion of carotenoids in higher plant photosynthetic apparatus in leaves (Osto *et al*., 2006). For this reason, green leaves have the potential to be used as a suitable source of lutein.

In this study, the level of lutein in *I. batatas* var. Oren leaves increased from 3.24 ±0.06 mg/ 100g FW and peak at 7.53 ±0.13 mg/ 100g FW during the leaves development. The study on the different pigments in kale also showed that the pigments levels varies with their development stages with the highest lutein level recorded at 15.1 mg/100 g in 1 to 2 weeks old of kale leaves(Lefsrud *et al*., 2007). Hence, this may suggest that the level of lutein in leaves is affected by their developmental stages. In this study, lowest level of lutein was observed during the first three days of leaf development and most probably because the young leaves are closed and therefore not active in photosynthesis. This is further confirmed by the lower chlorophyll level in young leaves when compared with the other developmental stages. However, as the leaves develop and

mature, the lutein content increases with increasing chlorophyll levels. Lutein level was observed at its highest level from day 9 to day 18. Besides, β-carotene content was also found to be highest in the Oren variety with the maximum concentration of 6.433 mg/100mg FW at day 15.

According to a research conducted by Ishiguro and Yoshimoto (2007), the amount of lutein detected in spinach was between 4.4 to 15.9 mg/100g FW. In a separate study, the contents of both lutein and β-carotene in spinach were found to be approximately 9.5 mg/100g FW and 8.9 mg/100g FW respectively (Khachik *et al*., 1992). This showed that the amount of lutein in *I. batatas* var. Oren leaves is comparable with the amount found in spinach. Currently, spinach and kale are ranked the highest among green leafy vegetables for their lutein contents (Torrey, 2011).

Previous studies on the β-carotene and lutein pigments have also been conducted on the leaves and edible portion of vegetables such as pepper, sesame and caper. Pepper leaves exhibited a lower β-carotene content compared to the *I. batatas* var Oren used in this study at 3.17 mg/100g FW whereas the lutein content was found to be higher at 8.69 mg/100g FW (Kim *et al*., 2007). Sesame leaves on the other hand contained lower pigments contents than the *I. batatas* var Oren with 2.58 mg/100g FW of β-carotene and 4.45 mg/100g FW of lutein (Kim *et al*., 2007). Besides that, study on the leaves of caper plant (*Capparis spinosa*) revealed that the leaves contained higher level of β-carotene and lutein pigments compared to their edible portion (Tlili *et al*., 2009). The pigments content in *I. batatas* also showed a similar pattern with the *I. batatas* storage roots exhibited lower pigments content (7.83 mg/100mg FW of β-carotene and 0.05 mg/g FW of lutein) compared to the *I. batatas* leaves studied in this chapter (Kidmose *et al.*, 2006). Comparatively, tomato fruits showed a total of 0.78 mg/100g FW of β-carotene and 19.6 mg/100g FW of lutein pigments (Kidmose *et al.*, 2006).

The relationships between the chlorophyll and lutein and β-carotene levels were also studied and the correlation provides a good prediction on the carotenoids levels in the leaves. However, so far, no study has been conducted on the correlation between chlorophyll and the carotenoids in other plants and therefore comparison cannot be performed. In this study, all the varieties showed high level of correlation between the chlorophyll and the carotenoids with the Oren and BK varieties having the highest correlation. This showed that the high level of chlorophyll is proportional to the level of carotenoids in leaves. Carotenoids are well known to have a major role in the photoprotection of chlorophyll from photooxidation in plants (Bartley and Scolnik, 1995). The presence of high levels of chlorophyll during the development of leaves is essential to facilitate the increase in food production through photosynthesis. Therefore, this could be the reason for the presence of high level of carotenoids when the chlorophyll level in the leaves is high.

Besides that, sample handling and storage is an important process after the collection of leaves sample prior to extraction. Good storage condition will ensure the quality and quantity of the pigments in the samples are retained. In this study, the best storage condition should retain the highest percentage of lutein and β-carotene pigments in a period of storage duration. The percentage of lutein and β-carotene remained in samples stored at 4ºC was found to be between the percentage for sample stored at room temperature and at 15ºC. Necrosis and leaves drying was only observed in leaves at day 6 of storage at 4ºC. During the first two days of storage, only slight decrease was observed in all varieties followed by larger decrement day 2 to day 6. At the sixth day of storage, the leaves contained approximately 30% of pigments remaining in their leaves and this amount was found to be higher compared to when stored in room temperature and 4°C. The same deterioration pattern was also observed for the β-

carotene pigments in the leaf sample. The leaves were observed to be in better condition after the same storage duration due to the inactivation of enzyme that causes deterioration when the samples were kept at lower temperature.

Comparatively, when the leaves were kept at room temperature (25ºC), the level of lutein in the leaves dropped significantly starting from the day of storage. This was followed by necrosis and drying of leaves from day 2 of storage onwards. The higher storage temperature contributes to a suitable breeding ground of fungus and microorganisms that causes destruction and damages to the leaves. During maturity, the chlorophyllic pigments decrease rapidly and virtually disappear as a part normal catabolic change in plant as the plant undergoes senescence (Leffingwell, 2002).

Although the carotenoids level also decreases during senescence, their degradation usually does not reach the point of extinction as compared to the chlorophyll pigments (Leffingwell, 2002). The lost in carotenoids can be partly due to the enzymatic oxidative degradation that occur in the plant itself and this degradation occur continuously throughout the life time of the plants and was not merely during the senescence stage (Leffingwell, 2002). Hence, 15°C was chosen as the best storage temperature for the leaves samples prior to the extraction process. The leaves also have a maximum storage of approximately 4 days before the pigments level deteriorate to below 80% of their total pigments. In addition, the Oren variety was chosen as the most suitable variety for extraction of natural yellow dye due to their higher durability during storage.

In a study conducted on blanched Brussels sprouts, storage at high temperature was found to decrease the nutritional value, odour and taste of the sprouts (Klimezka and Irzyniec, 1997). Besides, the degradation of anthocyanin pigments was also found to be

largely contributed by the storage temperatures (Withy *et al*., 1993). A study on the effect of storage temperature on the shelf life of Chinese cabbage revealed that large differences was observed between the vegetables stored in lower temperature (0ºC and 2ºC) and higher temperature (20ºC) with the latter resulted in shorter storage period for the vegetable. However, the presence of patchy papery necrosis was observed in the Chinese cabbage kept at 0ºC, which represents a form of chilling injury (Jiang and Pearce, 2005). Other factors that could contribute to the changes in samples materials include storage time, enzymatic and microbial changes.

Vegetables especially the green leafy one are often recommended to be stored in minimum temperature that can provide maximum shelf life. Besides, it was also documented in previous study that tropical vegetables such as the sweet basil had optimum storage temperature at 15ºC since chilling injury can occurred at lower temperature (Lange and Cameron, 1994). In many cases involving the tropical vegetables, ripening is never an issue but the storage temperatures were dependable upon the senescence process occurring at higher temperature and injury process at lower temperature.

As the conclusion, morphological variations in leaves can be used to identify and distinguish between the different *I. batatas* varieties and can be further supplemented by the storage roots morphologies. From the general screening of pigments, the *I. batatas* var. Oren leaves were chosen as the suitable material for the extraction of natural yellow dye due to the high concentration of lutein and β-carotene in the leaves. Besides, this variety also able to maintain the highest percentage of pigments during storage compared to the other varieties.

CHAPTER 4

Optimisation of Extraction Method and Stability of the Yellow Pigments Extracted from *Ipomoea batatas* Leaves

4.1 INTRODUCTION

Carotenoids extraction from oil palm, green leafy vegetables, alfalfa, seeds, alga (*Dunaleilla*), microalgae (*Scenedesmus almeriensis*) and bacteria (*Gemmatimonas aurantiaca*) are well documented in literature (Ben-Amotz and Avron, 1990; Sánchez *et al*. 2007; Takaichi *et al*, 2010). Carotenoids were extracted from various sources due to their antioxidant properties as well as their ability to possess colour ranging from yellow to red in nature (Ceron-Garvia *et al*., 2010). Furthermore, increasing consumers' preference for natural colourants coupled with government regulations has prompted the study on carotenoids extraction from natural sources. The extraction of carotenoids from natural sources such as plant materials usually includes a mixture of related pigments such as lycopene, zeaxanthin, capsanthin, canthaxanthin, phytoene and phytofluene, besides the two main carotenoid groups, carotenes and xanthophylls (Zelkha *et al*., 2004).

Carotenoids are lipophilic pigments in nature and therefore organic solvents are used for extraction. While different methods have been developed for the extraction of carotenoids from various sources, optimisation of the previously developed methods are important to maximise the amount of extracted carotenoids and reduce the duration and cost of extraction. Several parameters can contribute to the efficiency of the extraction method used, such as extraction solvents, concentration of potassium hydroxide used for

saponification and the duration for saponification. Since carotenoids in green leaves are often masked by chlorophyll pigments, saponification process is used to remove the chlorophyll from the leaf extracts.

In food industry, the stability of carotenoids against oxidation has been in the limelight due to nutritional and colour degradations that occur which reduces food quality (Minguez-Mosquera and Gandul-Rojas, 1994). Various studies have been conducted to determine carotenoid stability in the different storage conditions such as in spinach, carrot and vegetable juices (Pesek and Warthesan, 1987; Kopas-Lane and Warthesan, 1995). The rate and kinetics of the carotenoid degradation has been reported to be dependable on the storage conditions such as temperature, presence of light, water activity, oxygen, duration of storage and matrix characteristics (liquid or solid) (Chandler and Schwartz, 1987; Ceron-Garcia *et al.*, 2010). This study will focus on β-carotene and lutein, the two major pigments in leaves, which contribute to the natural yellow extract. Hence, the concentrations of these two pigments would be studied during the extraction process and stability experiments.

The first objective of this study is to determine the optimum conditions for the extraction of yellow pigments from *I. batatas* leaves. Secondly, is to determine the stability of the yellow pigments during storage in different conditions, which include factors such as matrix systems (acetone and soybean oil), illumination and temperature.

4.2 MATERIALS AND METHODS

4.2.1 Optimisation of Extraction Solvent

The extraction process of *I. batatas* var. Oren leaves were performed under low light condition. Firstly, the leaves were ground using liquid nitrogen. The finely ground powder (0.5 g) was then extracted with 50 ml of acetone in a 125 ml conical flask. The mixture was shaken overnight in an orbital shaker at room temperature (Stuart Scientific). Then, the extraction mixture was filtered with Whatman No.1 filter paper into a 50 ml Falcon tube, to which 0.1 g of butylated hydroxytoluene (BHT) was added. The remaining leaf powder was re-extracted with 20 ml of acetone according to the above-mentioned procedures. The filtrate was placed in ice to avoid pigments degradation. The extraction step was repeated using methanol, tetrahydrofuran and petroleum ether and tetrahydrofuran mixture (4:1) to replace acetone.

The solvent in the filtrate was removed through evaporation under vacuum using a rotary evaporator (Butchi). Then, 20 ml of 40% aqueous potassium hydroxide (KOH) was added into the extract and the mixture was left to saponify at 45ºC. Acetone (25 ml) was added to the saponified sample and the mixture was poured into a separating funnel. The bottom layer was discarded and the upper layer was collected into an evaporatory flask. The extract was evaporated using a rotary evaporator leaving a layer of oleoresin.

Next, 10 ml of petroleum ether and tetrahydrofuran mixture (4:1) was added to the oleoresin layer. A layer of green lipid will form at the base of the tube. The upper yellow layer was removed and used for analysis. Absorbance was measure at 480, 495, 645 and 655nm using a spectrophotometer. All the experiments were performed in

triplicates. The most suitable solvent for the extraction of the natural yellow dye was determined from the highest concentrations of β-carotene and lutein extracted, calculated from the equation provided in section 3.2.4.

4.2.2 Optimisation of Potassium Hydroxide (KOH) Concentration Used in Saponification

The method described in section 4.2.1 was used to determine the percentage of potassium hydroxide (KOH) used in the experiment. Acetone was determined as the best extraction solvent and therefore used in subsequent experiments. The different concentrations of potassium hydroxide (KOH) used were 10% KOH, 40% KOH and 60% KOH. The absorbance was recorded and the concentrations of β-carotene and lutein in the samples were calculated. The concentration of KOH used, which resulted in the highest amount of β-carotene and lutein extracted will be used in the subsequent extraction procedure.

4.2.3 Optimisation of Saponification Duration during Extraction

The method used for determining the optimum saponification duration was similar to section 4.2.1. From the result in section 4.2.2, 40% of KOH was found to extract the highest level of lutein and β-carotene pigments. In this experiment, acetone was used as extraction solvent and 40% of KOH was used to saponify the leaf extracts. Different saponification durations (30 min, 2 hours, 4 hours and overnight) were used to determine the best duration for the saponification process. The absorbance and concentration of β-carotene and lutein pigments in the sample was calculated. The duration of saponification used which extract the highest amount of β-carotene and lutein will be used in the extraction of the yellow pigments from the *I. batatas* leaves.

The summary of the parameters optimised are shown below:

Table 4.1: Parameters Used in the Optimisation of the Extraction of Yellow Pigments from *Ipomoea batatas* Leaves

Parameters		References
Types of extraction solvents	Acetone	Liu *et al.* (2007); Sánchez *et al*. (2008)
	Methanol	Wang and Liu (2009)
	Tetrahydrofuran (THF)	Arena *et al*. (2000)
	4 Petroleum Ether: 1 Tetrahydrofuran (THF)	Bulda *et al.* (2008)
Percentage of potassium hydroxide (KOH)	10% KOH	Morris *et al.* (2004)
	40% KOH	Chen *et al.* (2004)
	60% KOH	Nonier *et al*. (2004)
Saponification time	30 minutes	Sharpless *et al.* (2004)
	2 hours	-
	4 hours	Ng and Tan (1988)
	Overnight	Trujillo *et al.* (1990)

4.2.4 Stability of Yellow Pigments from *Ipomoea batatas* Leaves

4.2.4.1 Sample Preparation

Ten grams of finely ground *Ipomoea batatas* var. Oren leaves were extracted with 300 ml of acetone. The mixture was shaken overnight using an orbital shaker at room temperature. The following day, the mixture was filtered using a Whatman No. 1 filter paper, into a 500 ml Schott bottle and to the filtrate was added with 1 g of butylated hydroxytoluene (BHT). The leaves sample was re-extracted using 100 ml of acetone and filtered through a Whatman No. 1 filter paper. The filtrate was placed in ice and in the absence of light to avoid pigments degradation.

Next, the solvent from the filtrate was evaporated under vacuum using a rotary evaporator (Butchi). Then, 150 ml of 40% aqueous potassium hydroxide (KOH) was added and the mixture was left to saponify at 45ºC. Acetone (200 ml) was added to the saponified sample and the mixture was poured into a separating funnel. The bottom layer was discarded and the upper layer was collected in an evaporatory flask. The extract was evaporated using rotary evaporator leaving a layer of oleoresin.

Then, 150 ml of petroleum ether and tetrahydrofuran mixture (4:1) was added to the oleoresin. The green lipid layer formed at the bottom was removed and the upper layer was then evaporated. The yellow oleoresin formed was dissolved thoroughly in 420 ml of acetone. Next, 15 ml of the dissolved extract in acetone was placed into a 20 ml capped glass vial. The head space of the vial was filled with oxygen-free-nitrogen (OFN) gas to remove oxygen that can possibly cause degradation to the carotenoids

pigments. The cap of the vial was sealed with parafilm to avoid solvent evaporation (acetone is volatile solvent). A total of 28 vials were prepared and placed in different storage conditions.

The method above was repeated using soybean oil (Soya Lite, Malaysia) by substituting the usage of acetone with soybean oil. The sample extract was scanned from 380 nm to 700 nm wavelength using a spectrophotometer and the absorbances at 480, 495, 645 and 655 nm were determined. The formula used to calculate the lutein and β-carotene concentrations are as shown in section 3.2.4.

4.2.4.2 Stability of Extracted Yellow Pigments Stored in Different Conditions

The stability of the yellow pigments was studied under different storage conditions: (a) different storage temperatures, (b) presence or absence of light and (c) different matrices (acetone and soybean oil). The vials were kept at selected temperatures for fixed time intervals to study the pigments stability. The different storage conditions studied were: -20ºC (absence of light), 4ºC (absence and presence of light), room temperature or 25ºC (absence and presence of light) and 40ºC (absence and presence of light). For samples kept in the absence of light, the vials were covered with three layers of aluminium foil. All samples at the different conditions were prepared in triplicates. For short-term study, the sample were analysed periodically for one month while for the long-term study, analysis was conducted monthly over 6 months duration. The calculations for the lutein and β-carotene concentrations were shown in section 4.2.1.

4.3 RESULTS

4.3.1 Optimisation of Extraction Solvents

Figure 4.1 shows the picture of extracted sample using the different extraction solvents. Yellow coloured sample was observed from the extraction using acetone while pale yellow samples were obtained when methanol and petroleum ether and tetrahydrofuran mixture (4:1) were used. On the other hand, green coloured sample was observed when tetrahydrofuran was used as extraction solvent. The absorbance readings of the extracted samples were obtained and analysed.

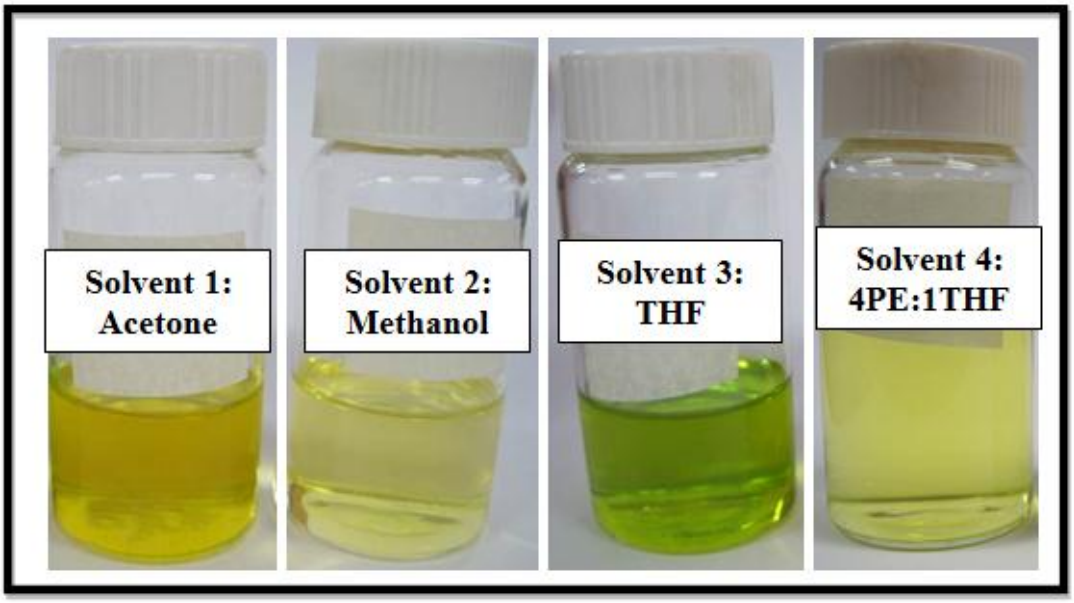


Fig 4.1 Yellow pigments extracted from *I. batatas* leaves using different extraction solvents

The concentrations of β-carotene and lutein were calculated and are shown in Figure 4.2. The highest concentration for both the β-carotene and lutein was observed with the usage of acetone as the extraction solvent. Methanol extracted the least amount of β-carotene and none lutein. Tetrahydrofuran (THF) ranked second as the best extraction solvent behind acetone followed by petroleum ether and tetrahydrofuran (4:1) mixture.

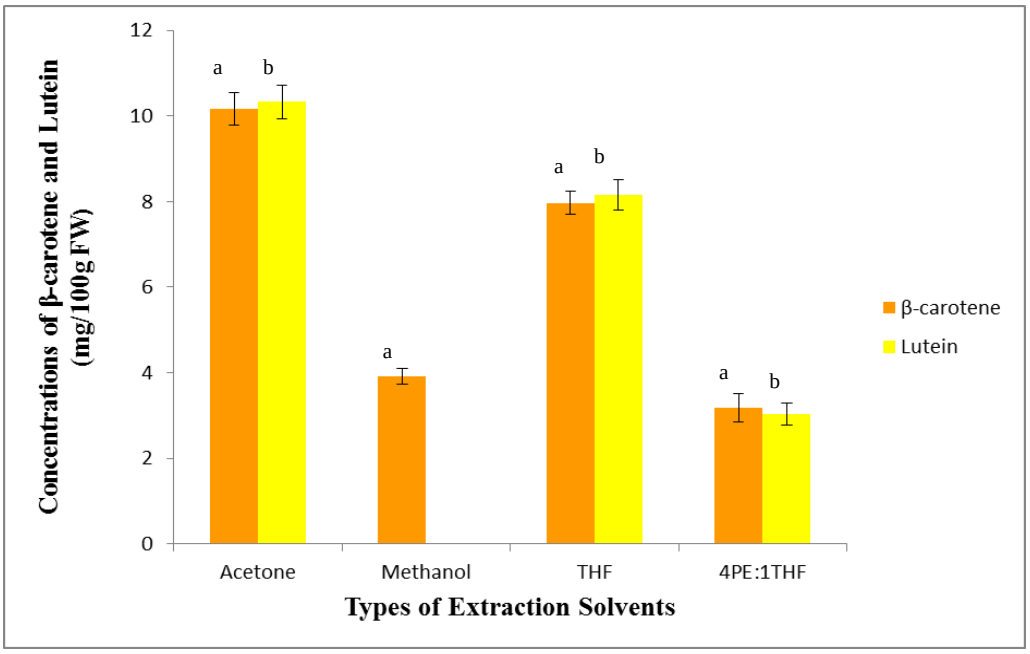


Fig 4.2 Concentrations of β-carotene and lutein in the yellow pigments extract using different extraction solvents

The similar letters above the columns indicate the statistically significant differences between the pigments concentrations when different extraction solvents were used at the $P<0.05$ level in Tukey's HSD test (n=6).

4.3.2 Optimisation of Potassium Hydroxide (KOH) Concentration Used in Saponification

The degree and efficiency of saponification process in an extract is dependable on the concentrations of KOH used which could adversely affect the colour and composition of the extracted product. Figure 4.3 shows the extracted sample using the different concentrations of KOH performed under mild light condition at room temperature. Extraction using 10% potassium hydroxide (KOH) did not successfully remove chlorophyll from the leaf extracts which was depicted by the absence of green ring on the extract surface and hence deemed unsuitable for subsequent experiment. A yellow-coloured sample was observed when 40% KOH was used in the saponification process while a brown-coloured sample was observed when 60% KOH was used in the saponification process.

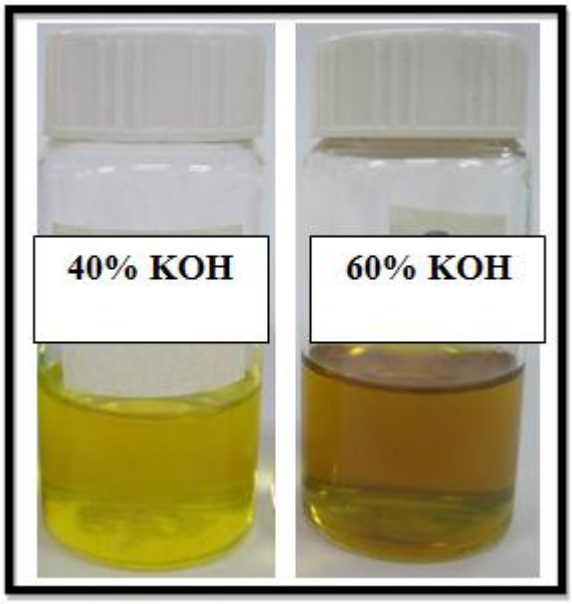


Fig 4.3 Yellow pigments extracted from *Ipomoea batatas* leaves using different concentrations of potassium hydroxide (KOH) during saponification

The results for the different concentrations of potassium hydroxide (KOH) used for saponification are shown in Figure 4.4. The usage of 40% KOH in the saponification process showed the highest extraction efficiency which can be obeserved from the high concentration of β-carotene and lutein extracted compared to the other KOH concentrations. As for the 60% potassium hydroxide, the level of both β-carotene and lutein extracted were lower compared to when 40% of KOH was used in the saponification process. Hence, 40% potassium hydroxide (KOH) was chosen as the best concentration for the saponification process.

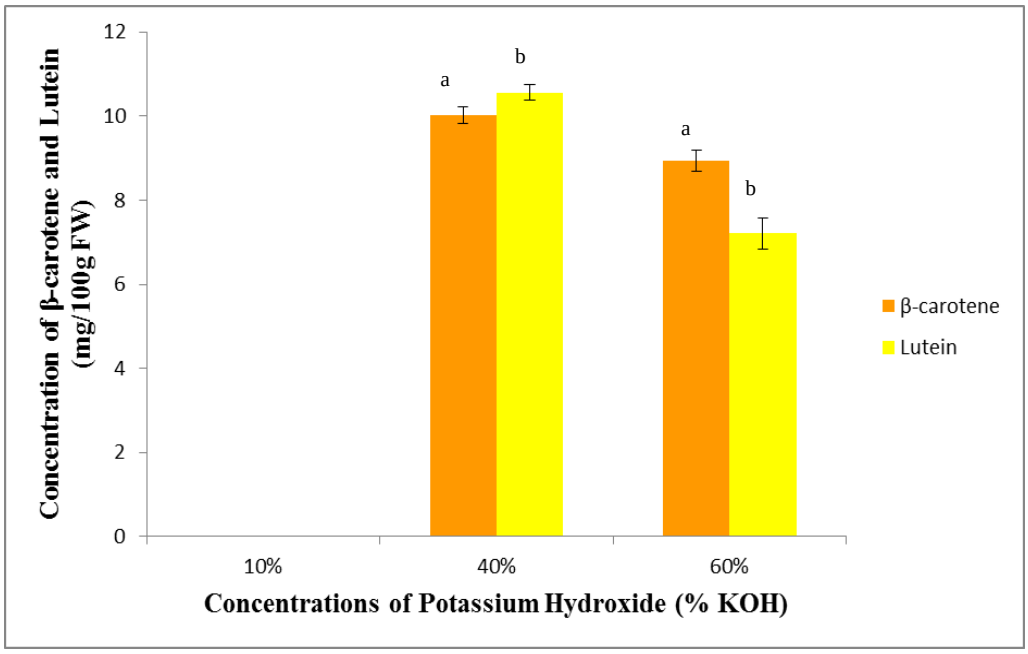


Fig 4.4 Concentrations of β-carotene and lutein in the yellow pigments extract using different concentrations of potassium hydroxide (KOH) for saponification

The similar letters above the columns indicate the statistically significant differences between the pigments concentrations when different concentrations of KOH were used at the $P<0.05$ level in Tukey's HSD test (n=6).

4.3.3 Optimisation of Saponification Duration during Extraction

The third factor studied was the determination of the optimum saponification duration for the extraction process. The sample extracts obtained using the different saponification durations at room temperature are shown in Figure 4.5. Yellow-coloured sample was observed for all the saponification durations used.

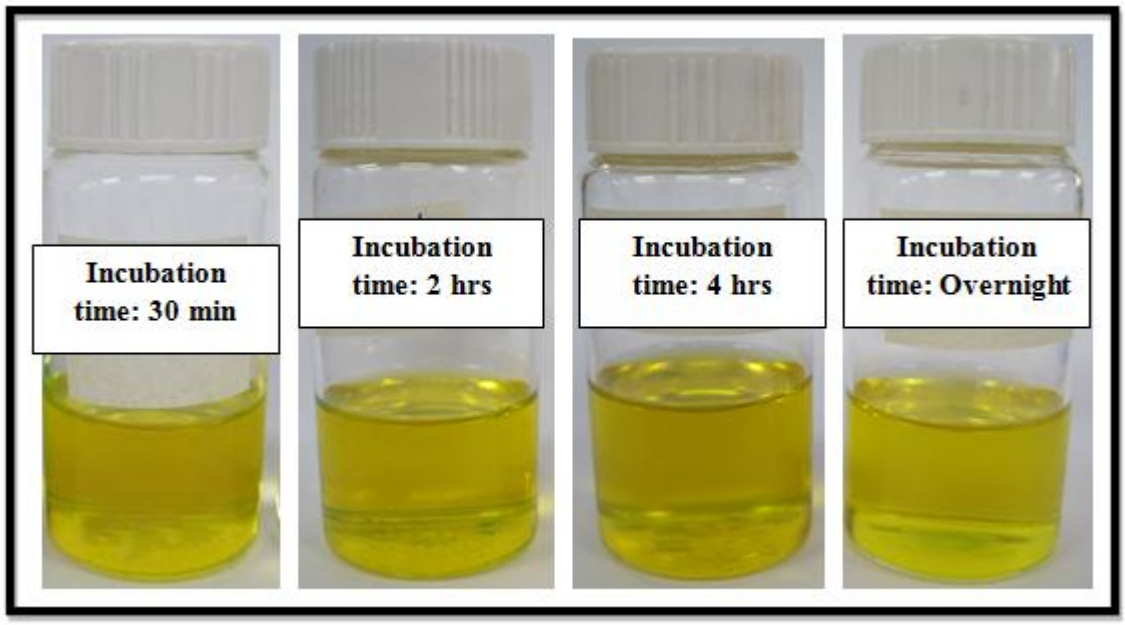


Fig 4.5 Yellow pigments extract from *I. batatas* leaves using different saponification durations

Figure 4.6 shows the concentration of β-carotene and lutein pigments when different saponification durations are used. Two hours and 4 hours saponification durations did not show significant differences in terms of the concentration of the extracted pigments. However, 30 min and overnight saponification yielded lower amounts of lutein compared to 2 hours and 4 hours. Hence, 2 hours of saponification was chosen as the best saponification length.

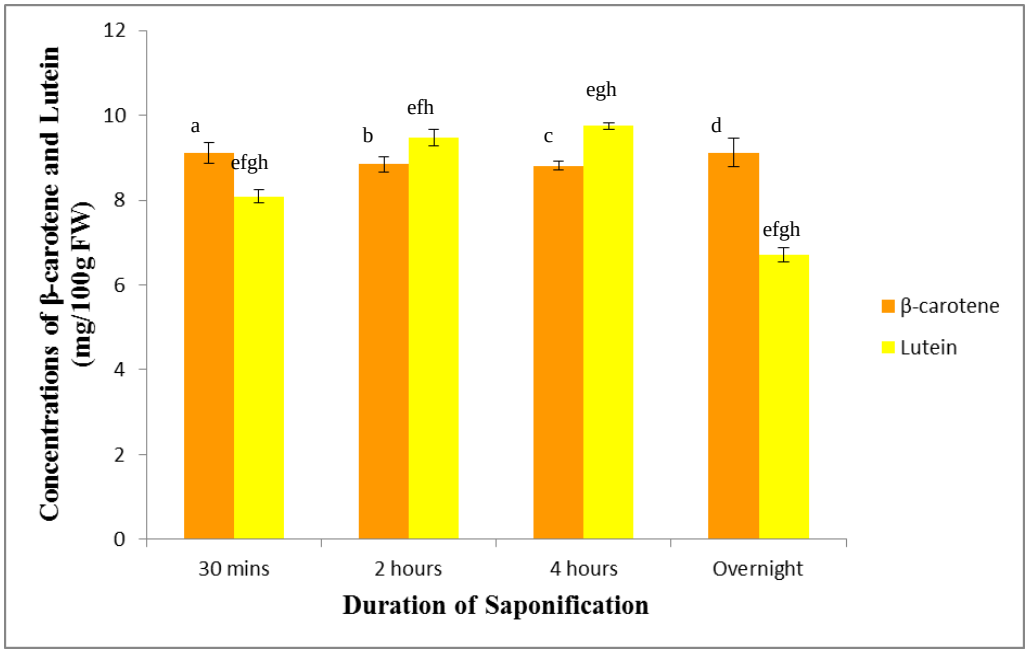


Fig 4.6 Concentrations of β-carotene and lutein in the yellow pigments extract using different saponification durations

The similar letters above the columns indicate the statistically significant differences between the pigments concentrations when subjected to different saponification duration at the $P<0.05$ level in Tukey's HSD test (n=6).

4.3.4 Stability of the Extracted Yellow Pigments from *Ipomoea batatas* Leaves

Scanning spectrophotometer was used to scan along the visible spectrum wavelength from 380nm to 700nm to determine the presence of carotenoid and chlorophyll pigments in the extract. The significant three peaks observed between 400 to 500nm confirmed the presence of carotenoids in the extract. Chlorophylls on the other hand if present, will be detected between 600nm to 700nm. Figure 4.7 shows the absorbance peaks for the yellow pigments in acetone. Three peaks were observed from the chromatogram at 432nm, 446nm and 472nm. No chlorophyll pigments were detected in the sample extract.

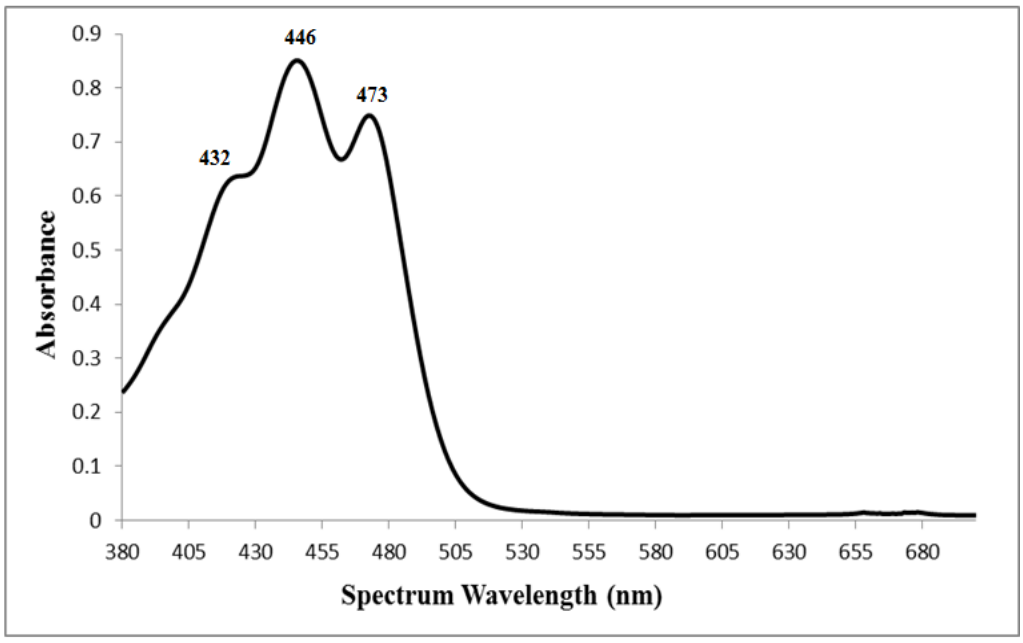


Fig 4.7 Graph of yellow pigments extract in acetone analysed using scanning spectrophotometer

Figure 4.8 shows the absorbance peaks for the yellow pigments extract in soybean oil. Three peaks were observed at 434nm, 448nm and 475nm wavelength, which were the characteristics peaks of the carotenoid pigments. No chlorophyll pigments was observed in the sample extract.

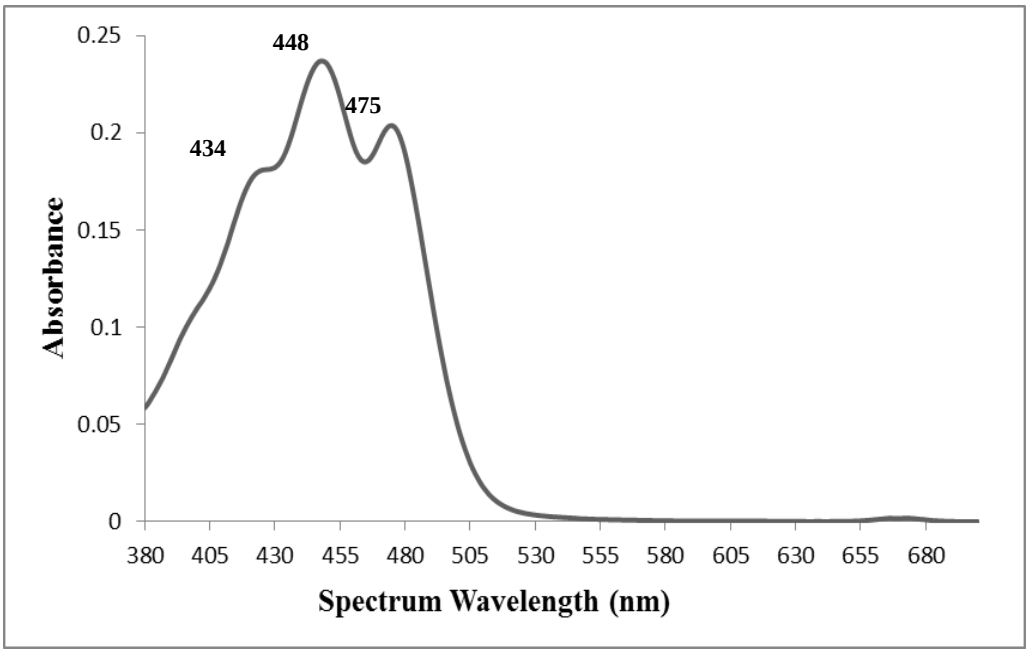


Fig 4.8 Graph of yellow pigments extract in soybean oil analysed using scanning spectrophotometer

Figure 4.9 shows a photograph of the yellow pigments extract from *I. batatas* leaves kept in both acetone and soybean oil. The sample extract was kept in a 20 ml capped glass vial.

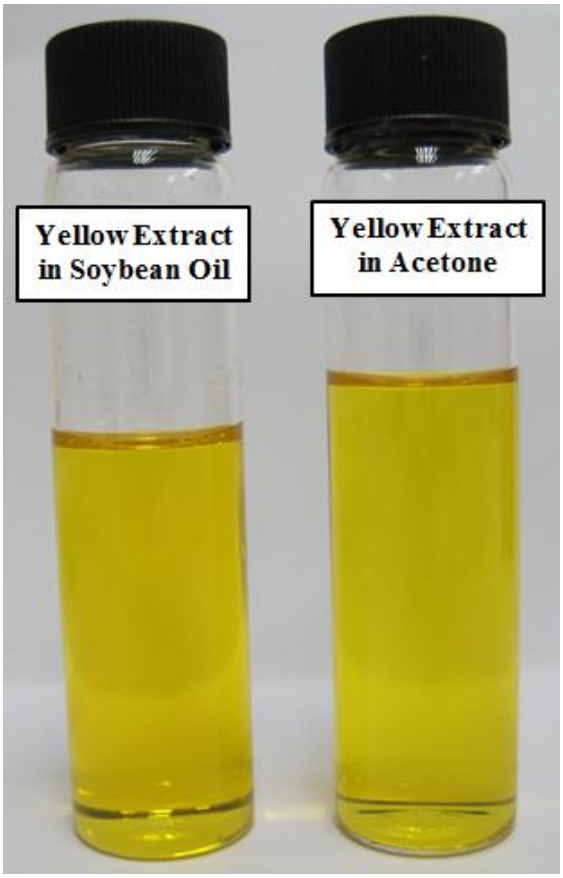


Fig 4.9 Picture of the yellow pigments extract in acetone and soybean oil

4.3.4.1 Short Term Analysis on the Stability of Extracted Yellow Pigments from *Ipomoea batatas* Leaves

The stability of the two main pigments: β-carotene and lutein were determined when stored in both acetone and soybean oil at -20°C for 8 days. Figure 4.10 shows the percentage of β-carotene and lutein pigments remaining in both the acetone and soybean oil from the day of extraction to the 8th days of storage. The β-carotene pigments showed least stability when kept in acetone with approximately 9% pigment degradation from day 2 to day 4 of storage. Lutein pigments in acetone and soybean oil however shows greater stability with approximately 95% remaining pigments after 8 days of storage.

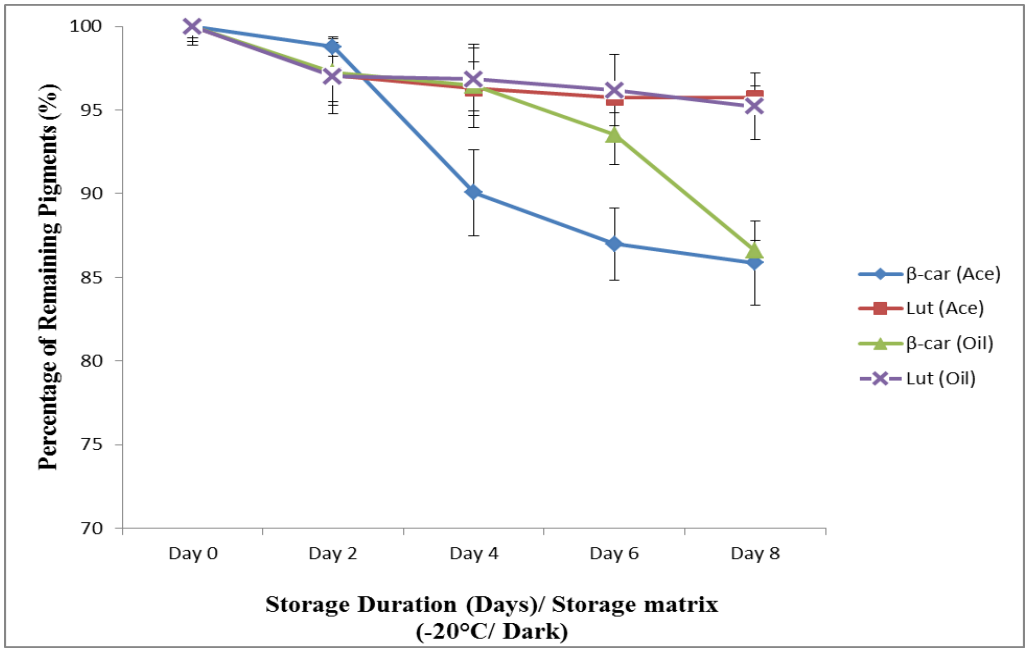


Fig 4.10 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in -20°C under dark condition (n=3)

Figure 4.11 shows the percentage of β-carotene and lutein pigments remaining in the sample kept in the dark at 4°C. Compared to storage at -20°C, lutein pigments kept in 4°C in both acetone and soybean oil showed greater instability. Overall, the β-carotene pigments in acetone showed greatest instability when kept at 4°C in the dark. However, β-carotene concentration after 8 days of storage was higher than when kept at -20°C.

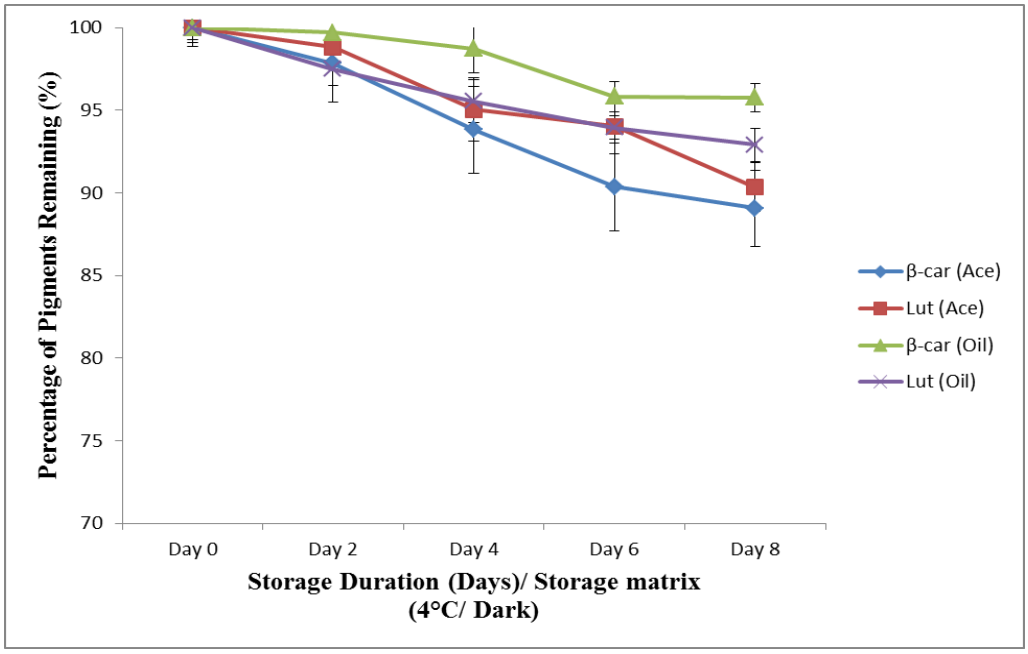


Fig 4.11 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in 4°C under dark condition (n=3)

Figure 4.12 illustrates the changes in the pigments concentration of the extract when kept at 4°C in the presence of light. Exposure to light at 4°C resulted in a large decrease (approximately 20% decrease from the initial pigments concentration) in both the lutein and β-carotene concentration kept in acetone after 8 days of storage. On the other hand, β-carotene and lutein pigments in soybean oil were more stable compared to when kept in acetone. This showed that illumination is an important factor that caused pigments degradation.

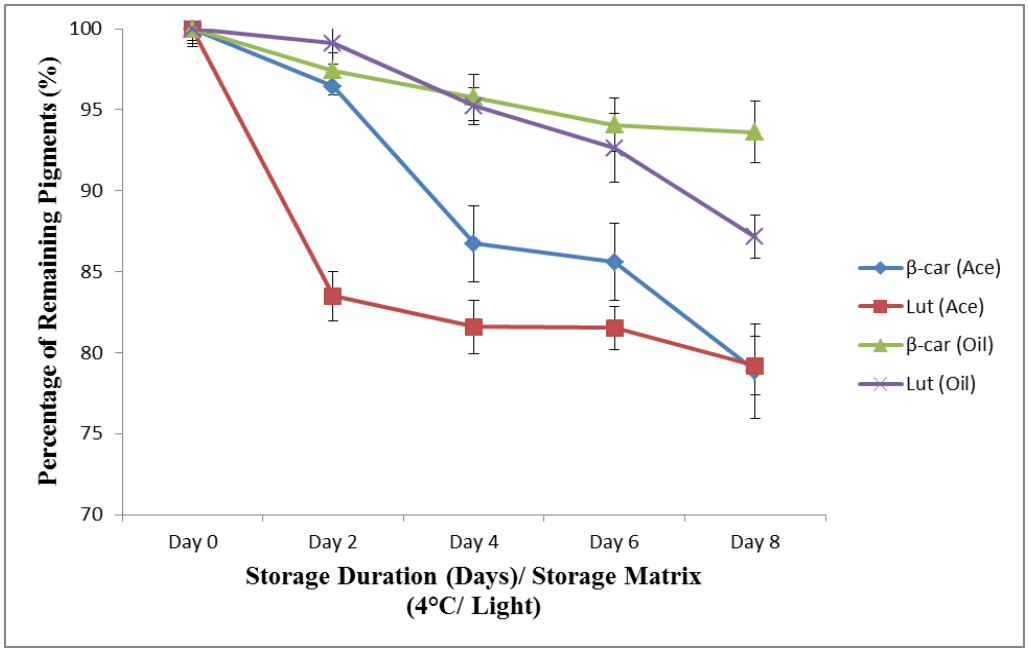


Fig 4.12 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in 4°C in the presence of light (n=3)

Figure 4.13 depicts the percentage of pigments in the sample kept at room temperature (25ºC) in the absence of light. A significant decrease was observed within the first two days of storage for the β-carotene pigments in acetone. The lutein pigments were found to have higher stability compared to the β-carotene pigments in soybean oil.

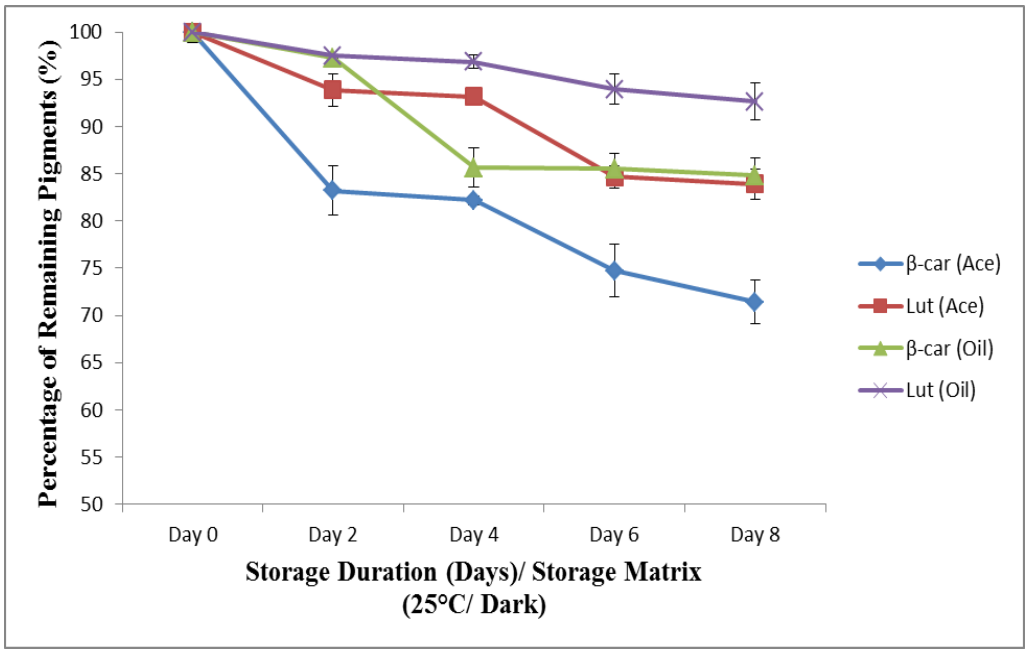


Fig 4.13 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in 25°C under dark condition (n=3)

The percentage of β-carotene and lutein pigments in the sample kept at room temperature (25ºC) in the presence of light is shown in Figure 4.14. Lutein pigments in acetone showed greatest instability with approximately 40% of pigments degradation occurred after 8 days of storage. Storage in soybean oil brought about higher stability for both the β-carotene and lutein pigments compared to when kept in acetone.

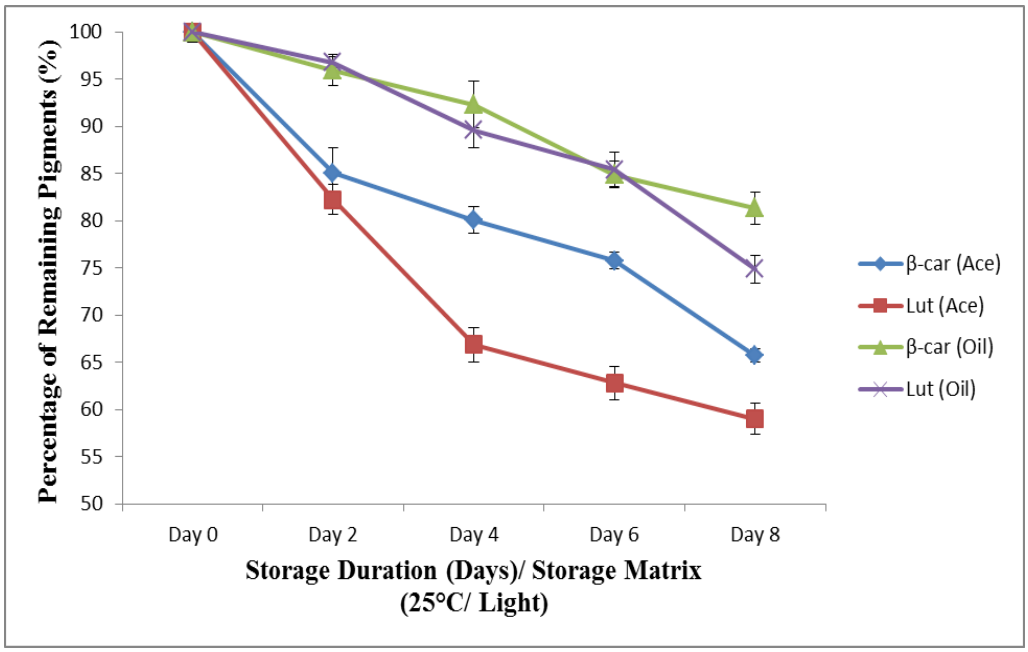


Fig 4.14 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in 25°C in the presence of light (n=3)

Storage at higher temperature (40°C) brought greater instability to the pigments with significant decrease observed for both the β-carotene and lutein pigments kept in acetone as shown in Figure 4.15. Comparatively, the loss of pigments was less severe compared to storage at 25°C with the presence of light. Pigments stored in soybean oil medium was found to be more stable compared to storage in acetone.

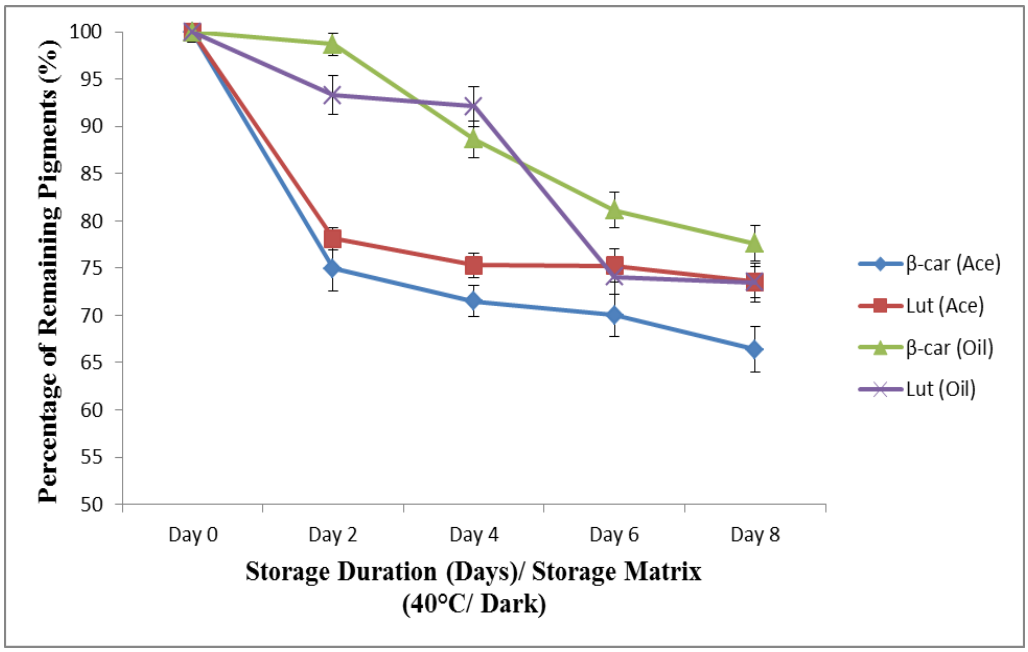


Fig 4.15 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in 40°C under dark condition (n=3)

Figure 4.16 illustrates the stability of the β-carotene and lutein pigments in samples kept at 40°C in the presence of light. Significant loss was observed for both β-carotene and lutein pigments kept in acetone with the former showed approximately 45% pigments degradation. Larger loss of pigments was observed compared to storage at 25°C (light) and 40°C (dark) conditions. Hence, it can be concluded that light contributes to instability of the pigments while higher storage temperature further aggravated the degradation of these pigments.

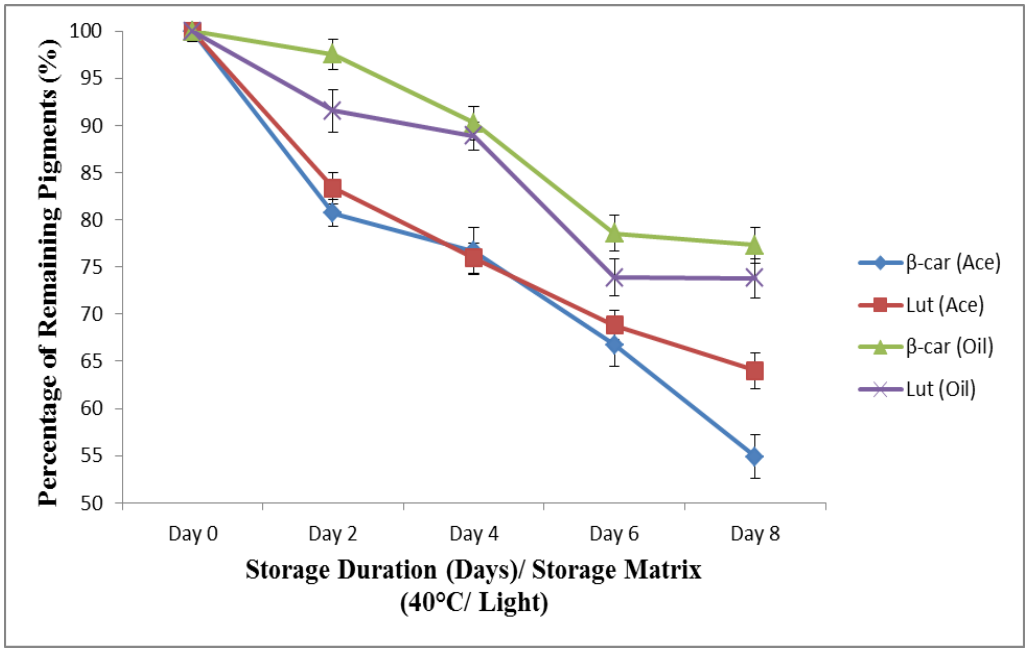


Fig 4.16 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in 40°C in the presence of light (n=3)

The stability of the β-carotene and lutein pigments in acetone and soybean oil kept in different conditions for 8 days duration are summarised in Figure 4.17. It was observed that the presence of light promote greater instability for the β-carotene pigments in the sample. Surprisingly, storage at 4°C in the absence of light showed higher stability of pigments compared to storage at -20°C. Storage of sample at 40°C in the presence of light shows the greatest pigments degradation after 8 days of storage.

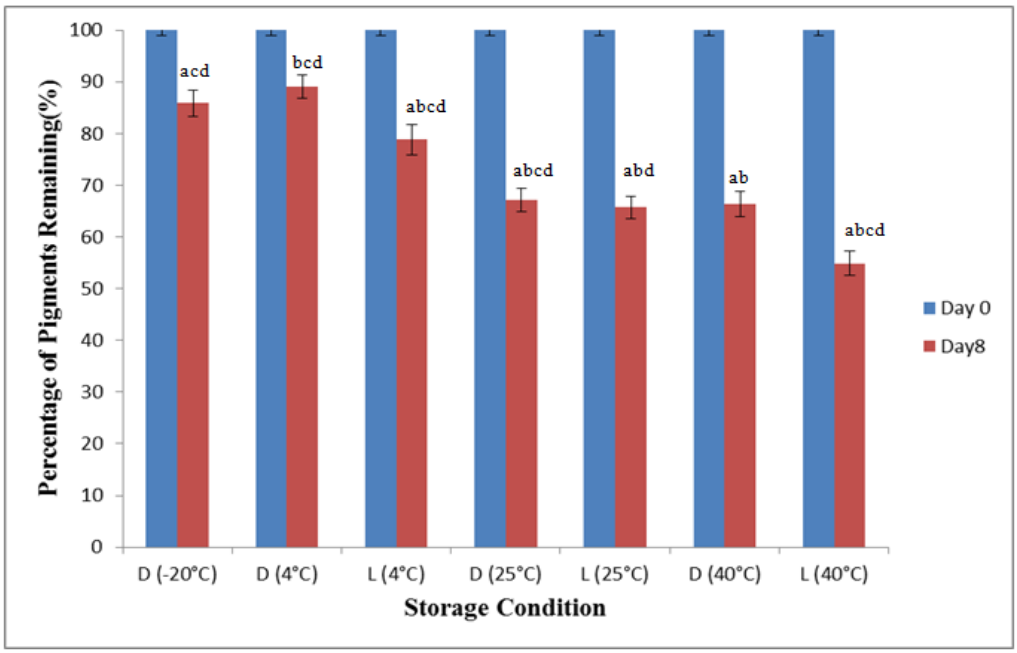


Fig 4.17 Percentage of β-carotene pigments in acetone when kept at different storage conditions (n=3)

The similar letters above the columns indicate the statistically significant differences between the pigments concentrations at the $P<0.05$ level in Tukey's HSD test (n=3).

Figure 4.18 shows the percentage of lutein pigments remaining in acetone after 8 days of storage. The greatest lost of pigments was observed when sample was kept at room temperature (25°C) in the presence of light followed by sample at 40°C (in the presence of light). It was observe that light decrease the stability of the lutein pigments in this study.

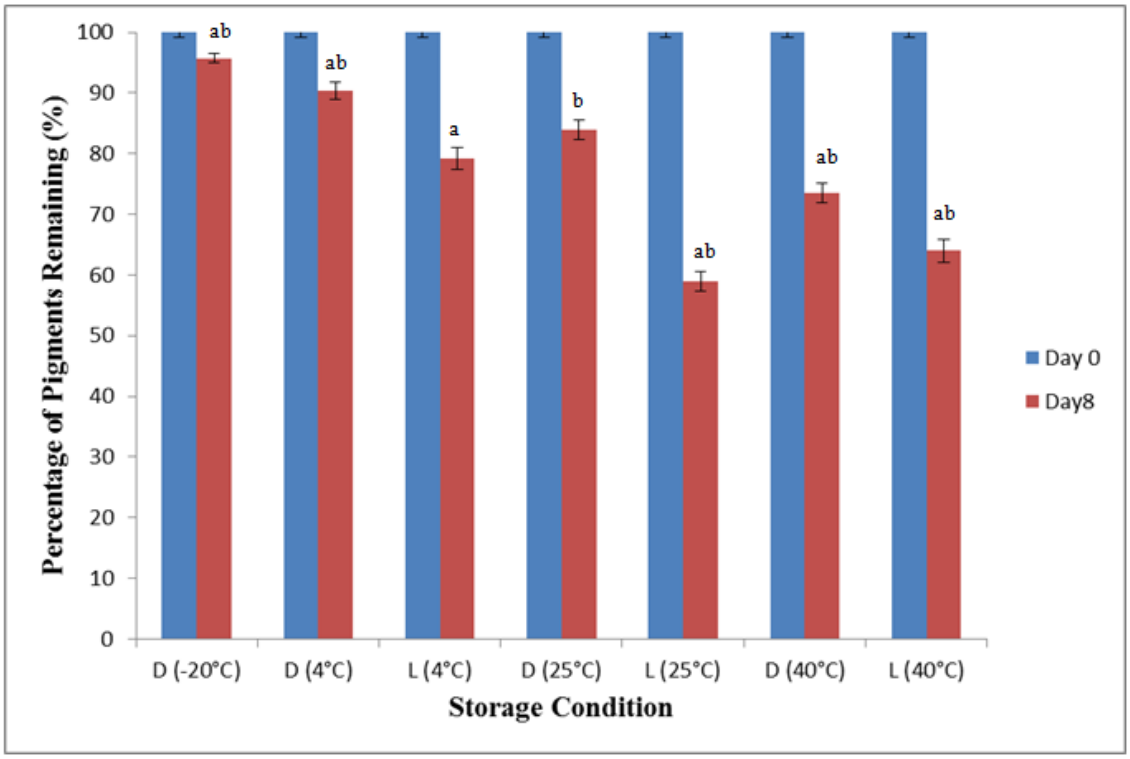


Fig 4.18 Percentage of lutein pigments in acetone when kept at different storage conditions (n=3)

The similar letters above the columns indicate the statistically significant differences between the pigments concentrations at the P<0.05 level in Tukey's HSD test (n=3).

Figure 4.19 shows the percentage of the β-carotene pigments in soybean oil kept at different storage conditions. No significant difference was observed for the loss of pigments between storage at 25°C and 40°C in the presence or absence of light. However, storage at -20°C showed lower instability compared to at 4°C. The presence of light however does not significantly caused the degradation of pigments in the soybean oil. Higher temperatures brought about greater instability compared to when stored in lower temperature.

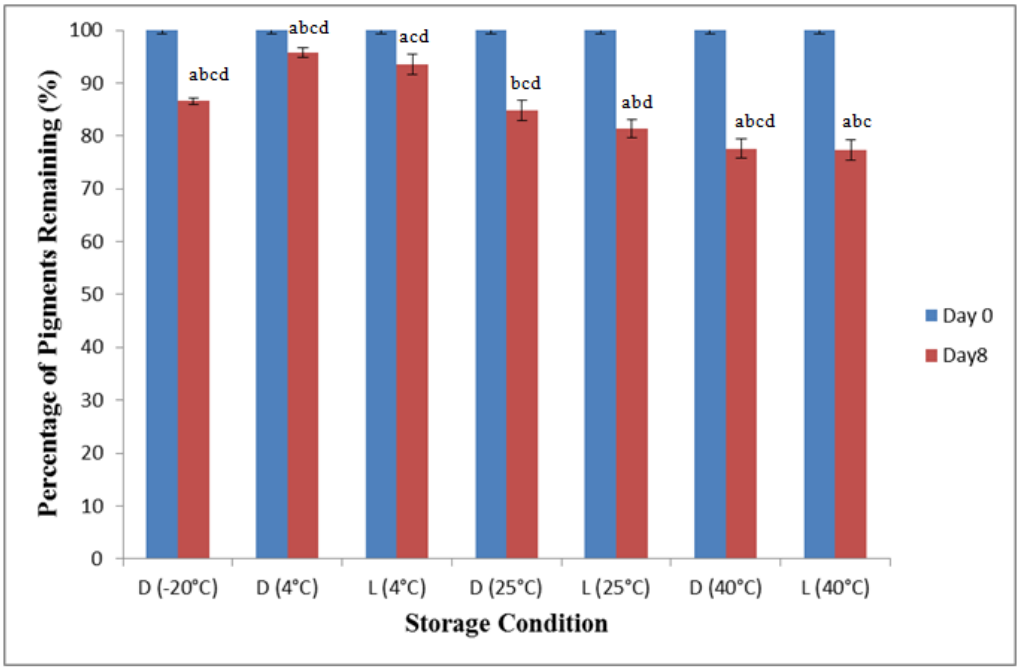


Fig 4.19 Percentage of β-carotene pigments in soybean oil when kept at different storage conditions (n=3)

The similar letters above the columns indicate the statistically significant differences between the pigments concentrations at the $P<0.05$ level in Tukey's HSD test (n=3).

Figure 4.20 shows the percentage of lutein pigments in soybean oil kept at different conditions. Samples stored at -20°C (dark), 4°C (dark) and 25°C (dark) showed no significant difference after 8 days of storage. However, larger pigment loss was observed for samples kept in 25°C (light), 40°C (dark) and 40°C (light) conditions. Hence, high temperature and light both are important factors that cause pigment degradation in the samples.

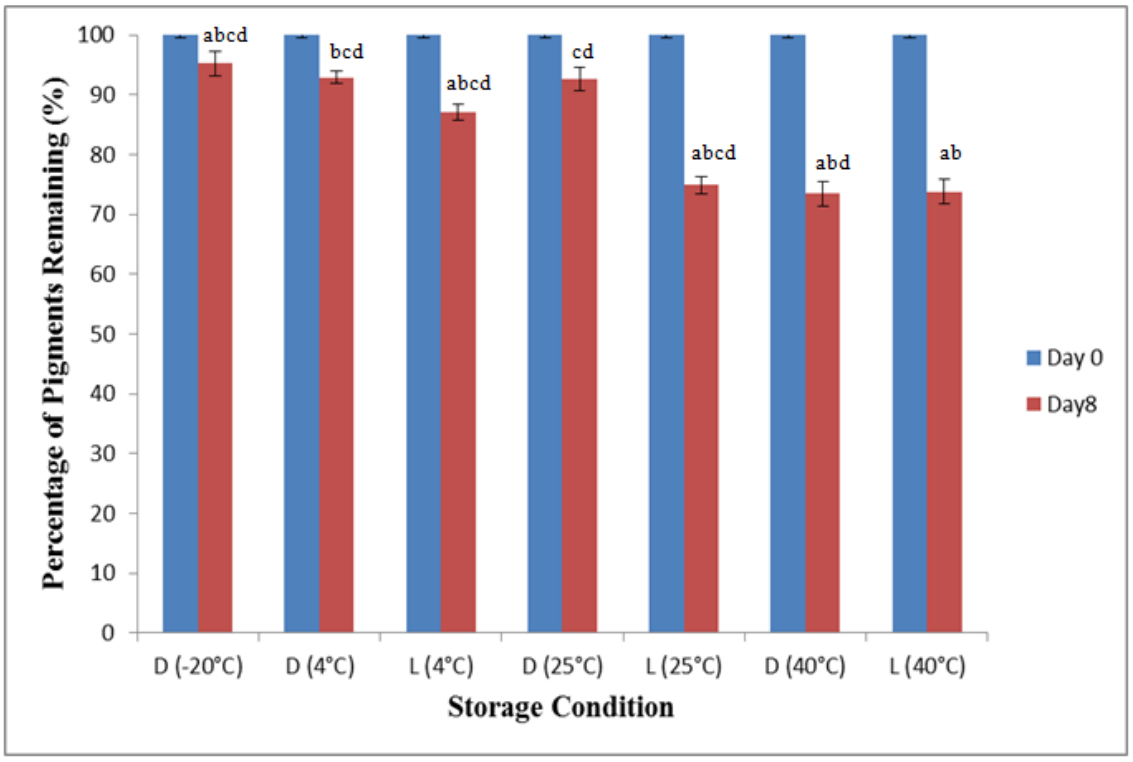


Fig 4.20 Percentage of lutein pigments in soybean oil when kept at different storage conditions (n=3)

The similar letters above the columns indicate the statistically significant differences between the pigments concentrations at the $P<0.05$ level in Tukey's HSD test (n=3).

4.3.4.2 Long Term Analysis on the Stability of Extracted Yellow Pigments from *Ipomoea batatas* Leaves

The long-term stability for both the β-carotene and lutein pigments in the yellow pigments extract from *Ipomoea batatas* leaves was determined throughout six months period. Figure 4.21 shows a photograph of the extracted natural yellow dye sample after six months of storage at -20°C. Samples stored in acetone and soybean oil retained the yellow colour after six months of storage.

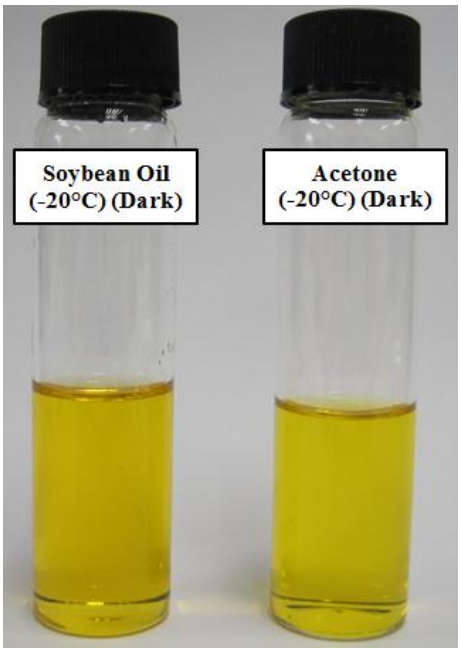


Fig 4.21 Picture of the yellow pigments extract in acetone and soybean oil after six months of storage in -20°C (Dark)

Figure 4.22 shows the percentage of β-carotene and lutein pigments remaining in the sample during six months of storage. The β-carotene pigments in acetone and soybean oil showed greater instability compared to lutein throughout the six months storage period. Significant loss of β-carotene pigments was observed when kept in acetone compared to in soybean oil and the levels continue to decrease to the sixth month of storage. Lutein pigments in soybean oil showed larger pigments loss during the first month of storage followed by a stagnant concentration until the sixth month of storage.

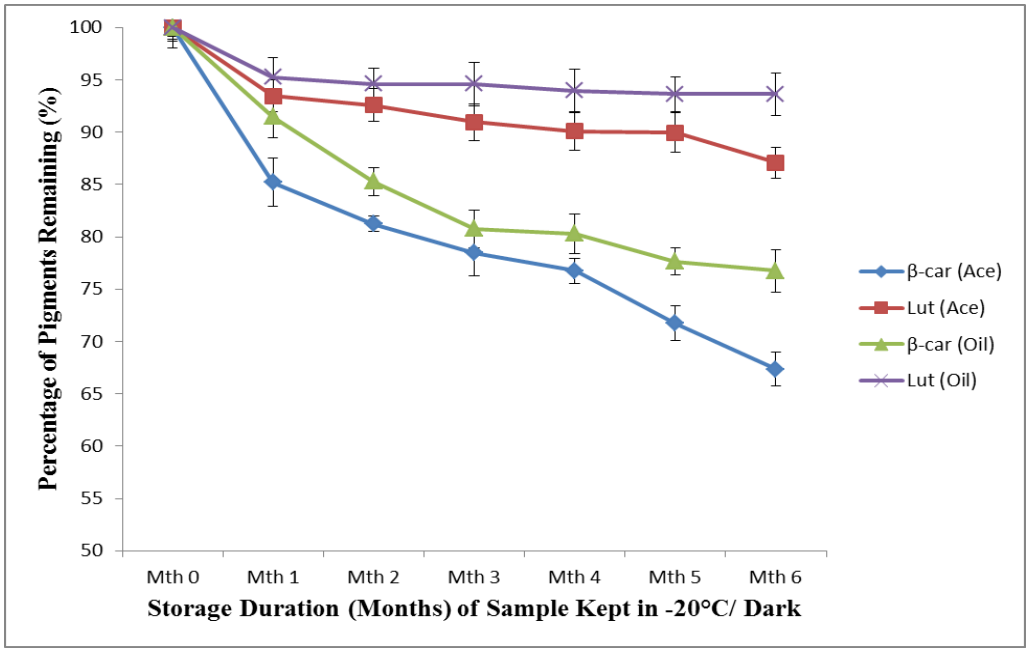


Fig 4.22 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in -20°C in the dark (n=3)

Figure 4.23 shows a photograph of the yellow pigments extract kept in 4°C after six months of storage. Pigments degradation occurred in sample stored in acetone (in the presence of light) which resulted in the discolouration of the sample. However, the samples in acetone (absence of light) and soybean oil retained the yellow colour after six months of storage.

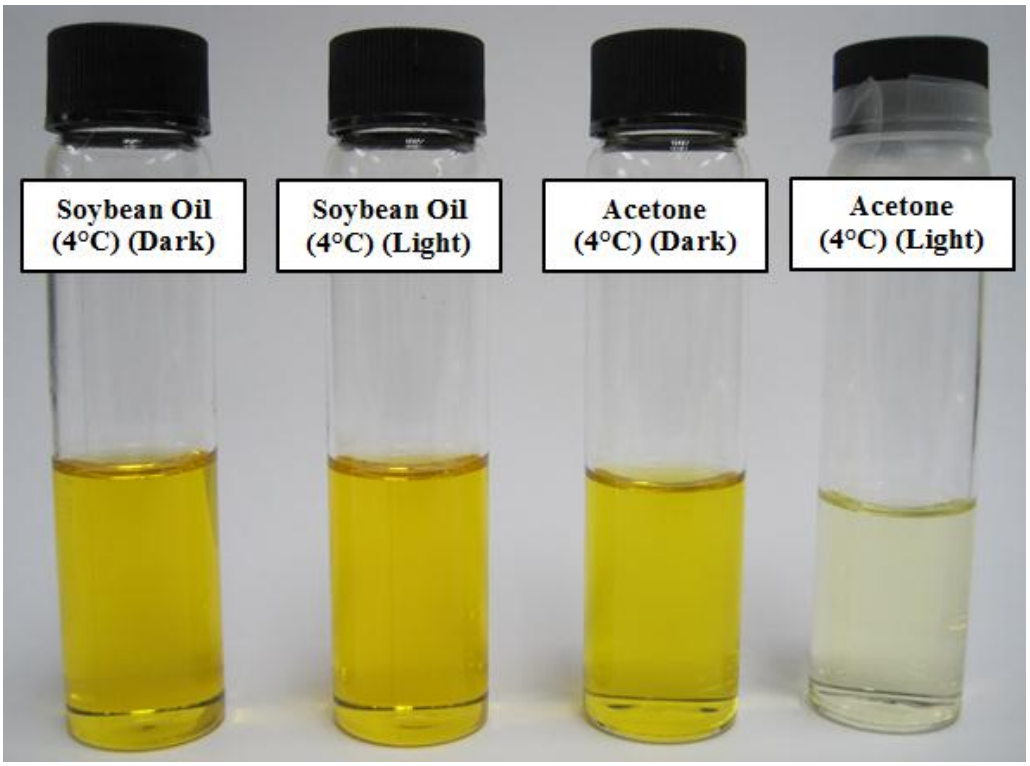


Fig 4.23 Picture of the yellow pigments extract in acetone and soybean oil after six months of storage at 4°C

Figure 4.24 shows the percentage of pigments remaining in the yellow pigments extract during six months storage at 4°C in the dark. The largest pigments loss was observed for lutein in acetone during the first 4 months of storage and the stability was maintained thereof. The lutein and β-carotene pigments in soybean oil however, showed almost similar pattern of pigment loss and their differences were not significant.

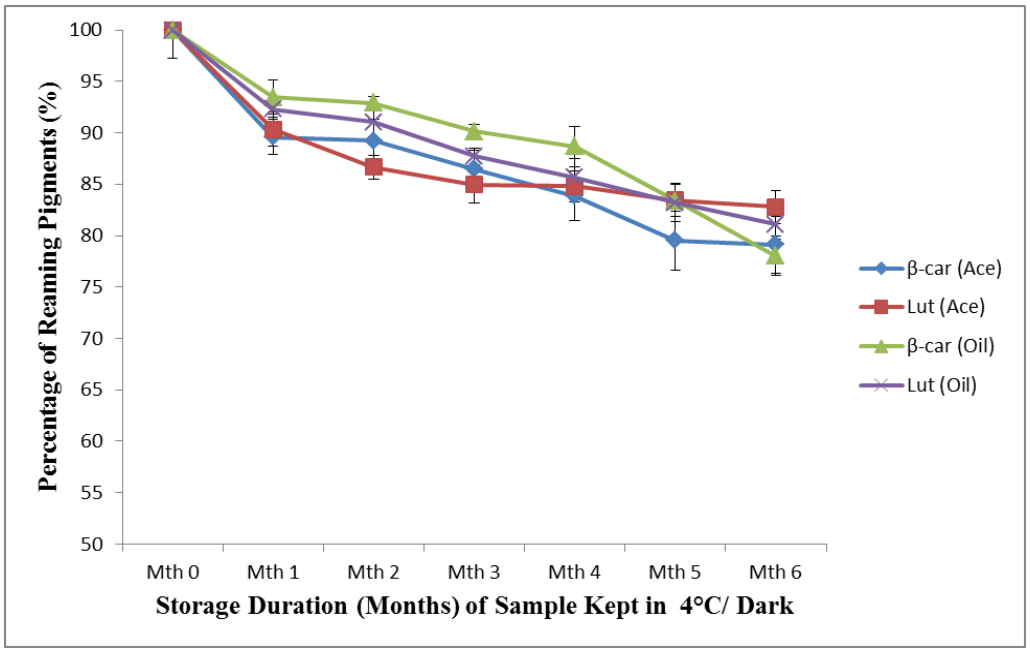


Fig 4.24 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in 4°C in the dark (n=3)

Figure 4.25 shows the percentage of β-carotene and lutein in sample kept at 4°C for six months duration. Generally, both the β-carotene and lutein pigments in soybean oil are more stable with approximately 77% of β-carotene and 73% of lutein remaining in the sample after 6 months of storage. However, the β-carotene and lutein pigments in acetone dropped drastically from the first month of storage and complete degradation of pigments was observed on the fifth months of storage.

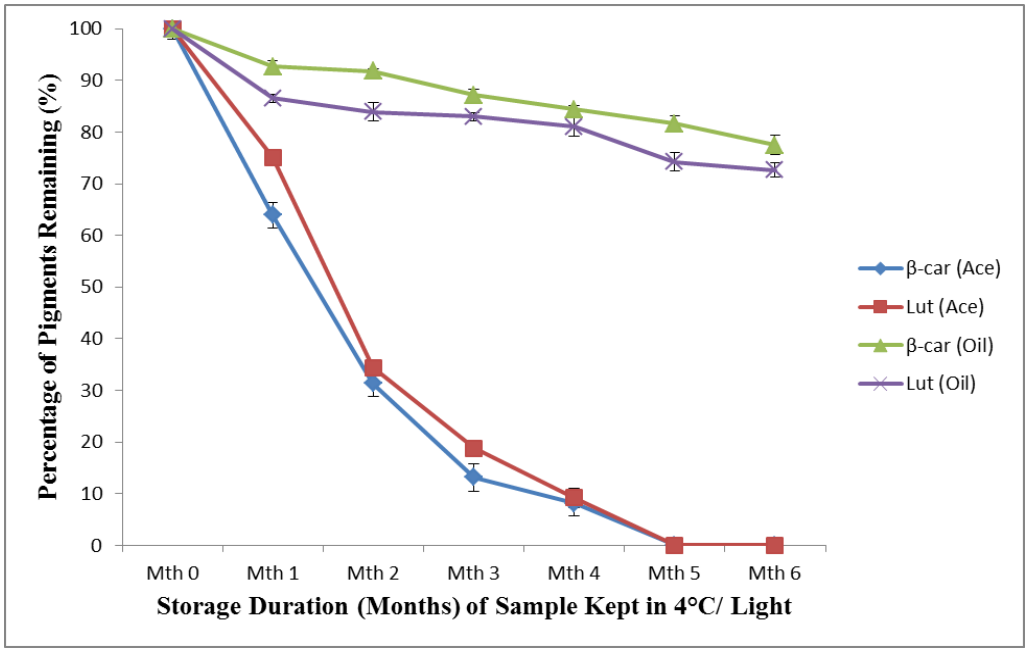


Fig 4.25 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in 4°C in the presence of light (n=3)

Figure 4.26 shows the photograph of the yellow pigments extract after six months storage at 25°C (room temperature). Total pigments degradation occurred in sample stored in acetone (in the presence of light) which can be observed by the discolouration of the sample colour to transparent. The sample in soybean oil and acetone (in dark) remained yellow after six months of storage duration.

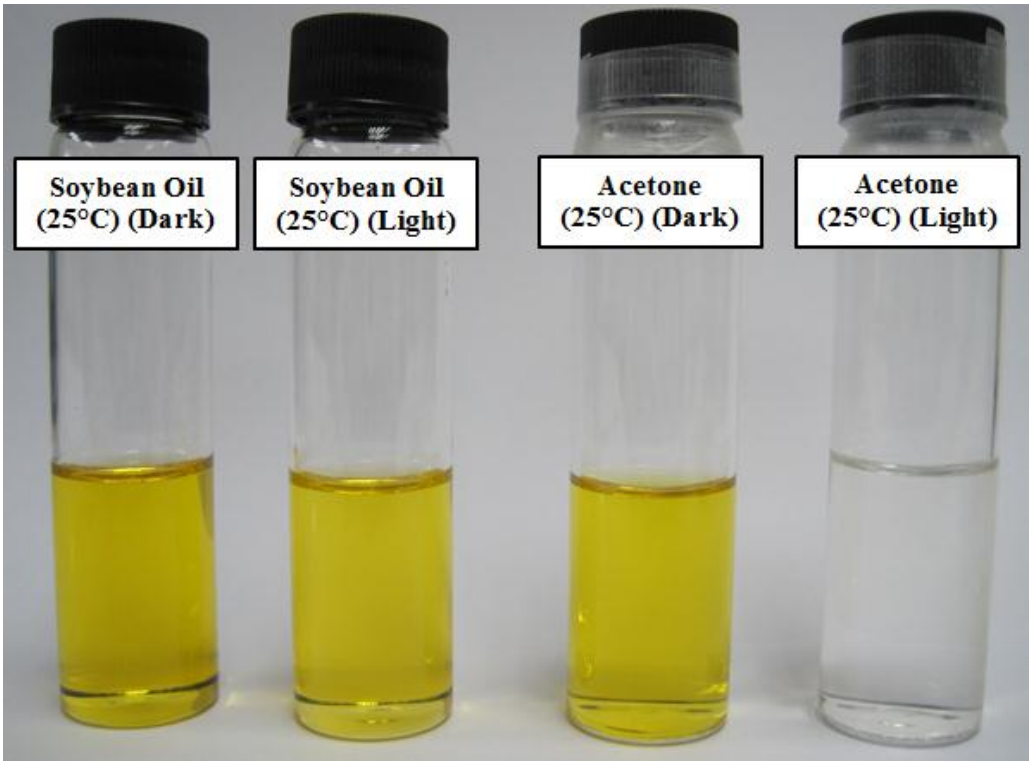


Fig 4.26 Picture of the yellow pigments extract in acetone and soybean oil after six months storage in 25°C (room temperature)

Figure 4.27 shows the percentage of the β-carotene and lutein pigments in sample kept at room temperature (25°C) in the absence of light for six months duration. Largest decrement was observed during the first month of storage for all samples. β-carotene pigments in acetone showed the largest pigments loss (60% loss) after six months of storage. The β-carotene pigments in soybean oil and lutein pigments in acetone showed almost similar patterns of pigents loss. The lutein pigments in soybean oil showed highest stability during the six months storage duration.

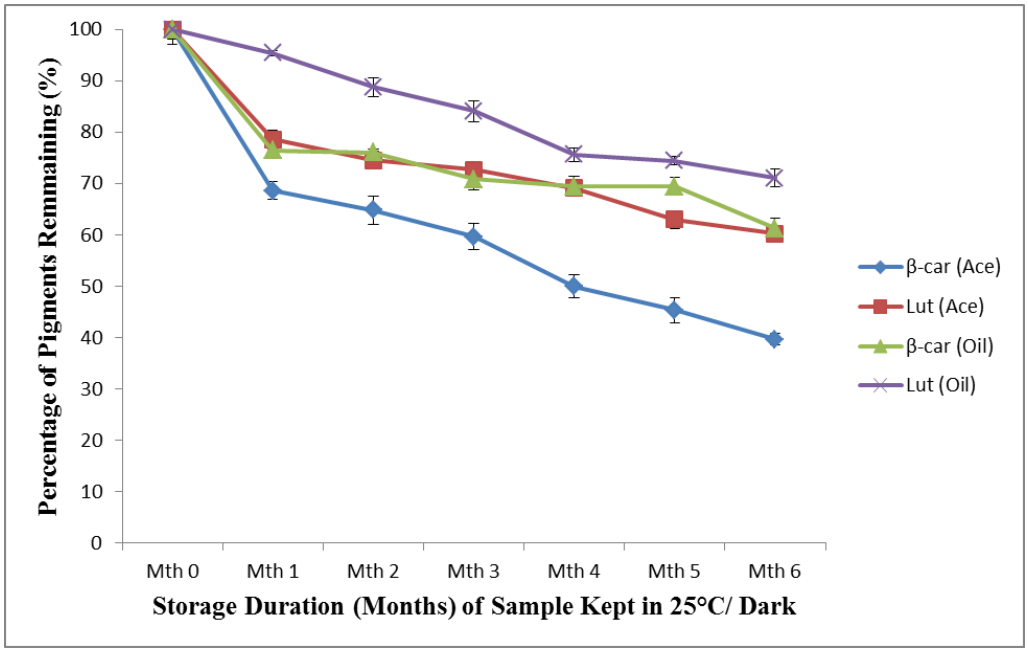


Fig 4.27 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in 25°C in the dark (n=3)

Figure 4.28 shows the percentage of the β-carotene and lutein pigments in sample stored at 25°C (in the presence of light). The lutein pigments in acetone drop dramatically during the first two months of storage which resulted in total pigments loss. The β-carotene pigments on the other hand also dropped significantly during the first two months of storage and total loss of pigments was observed in the fourth month of storage. A steep decrease was observed in both the β-carotene and lutein pigments when the sample was stored in soybean oil during the first month of storage followed by slight decrease from the second month onwards.

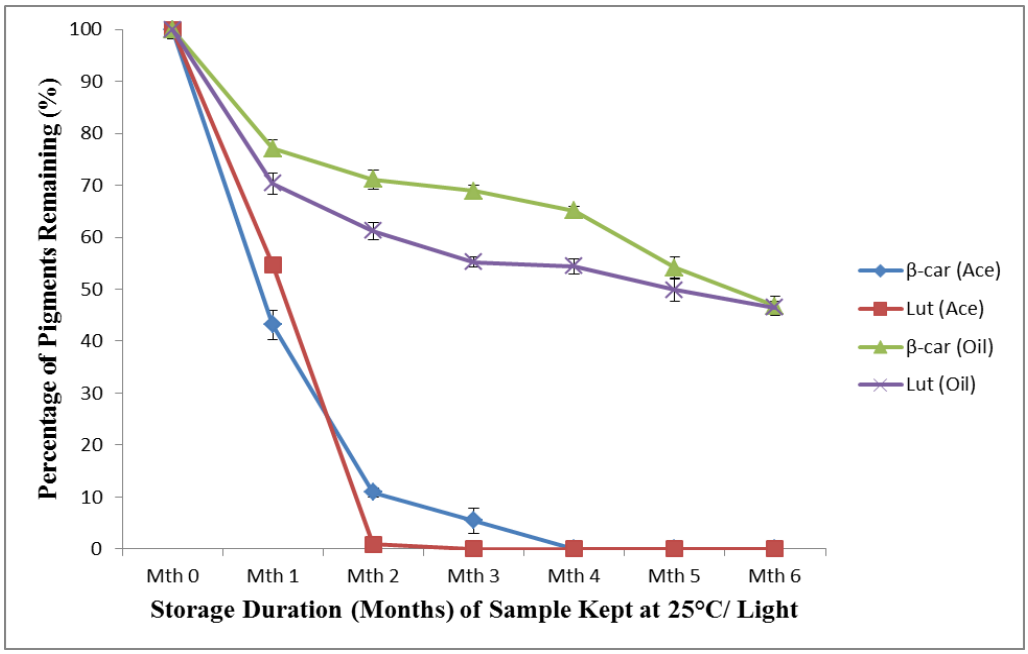


Fig 4.28 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in 25°C in the presence of light (n=3)

Figure 4.29 shows a photograph of the yellow pigments extract after six months of storage duration at 40°C. The sample in acetone decolourized and turned transparent upon storage in the presence of light. Sample in acetone stored in the dark however only showed slight discolouration in its colour. Nevertheless, samples kept in soybean oil showed better stability compared to in acetone.

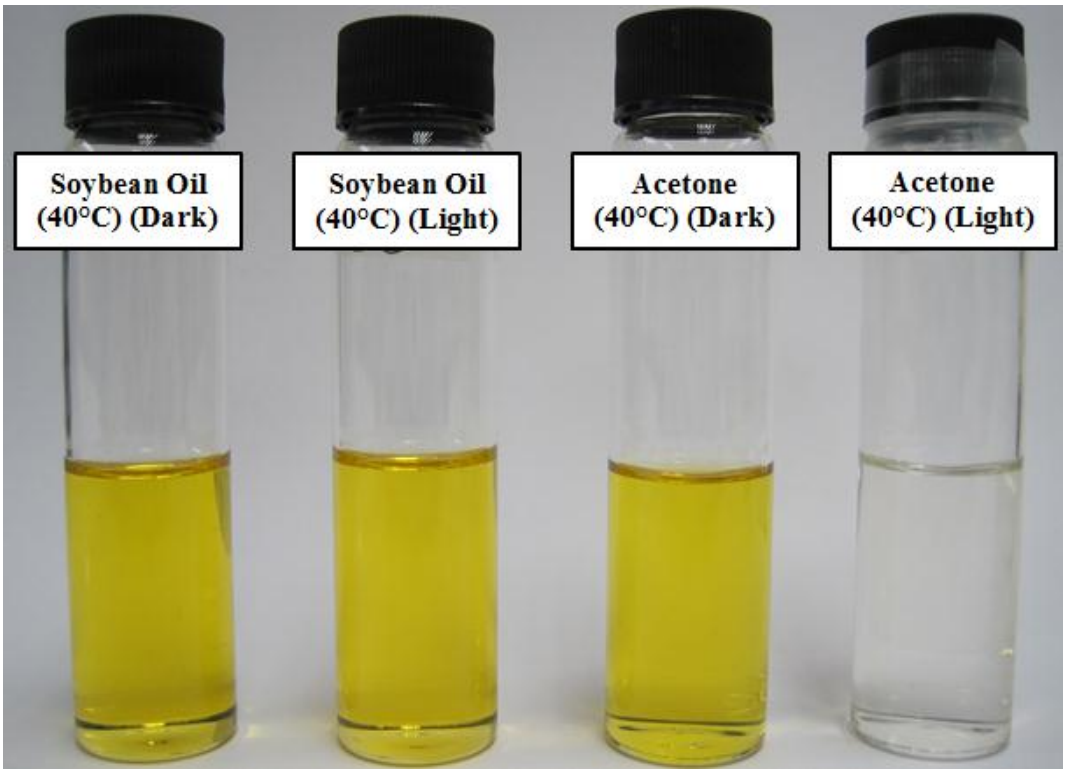


Fig 4.29 Picture of the yellow pigments extract in acetone and soybean oil after 6 months storage in 40°C

Figure 4.30 shows the percentage of β-carotene and lutein pigments in samples stored at 40°C during a six month storage period. The first month of storage recorded the largest loss of pigments for all the samples. The β-carotene pigments in acetone showed least stability with approximately 37% of pigments remaining after six months of storage. Pigments in soybean oil were found to be more stable compared to in acetone.

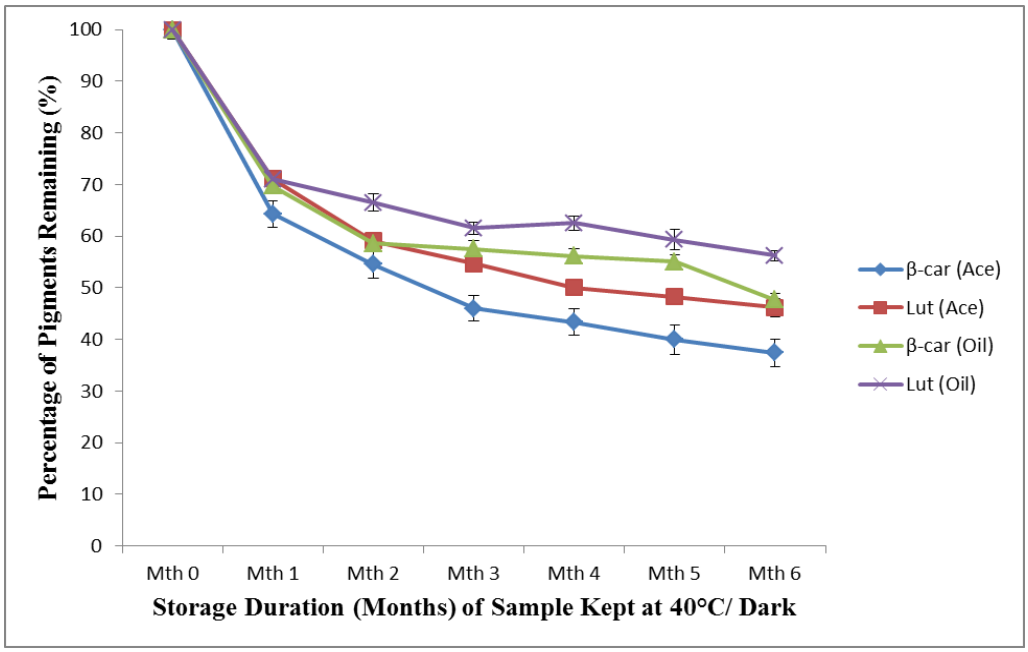


Fig 4.30 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in 40°C in the dark (n=3)

Figure 4.31 shows the percentage of the β-carotene and lutein pigments in samples kept in 40°C in the presence of light. Total loss of lutein pigments was observed during the first month of storage in acetone. The β-carotene pigments in acetone dropped significantly during the first month of storage and total loss of pigments was observed in the following month. The total loss of pigments in sample can be observed by the discolouration of sample colour during storage. On the other hand, both the β-carotene and lutein pigments in soybean oil were found to be more stable compared to when stored in acetone.

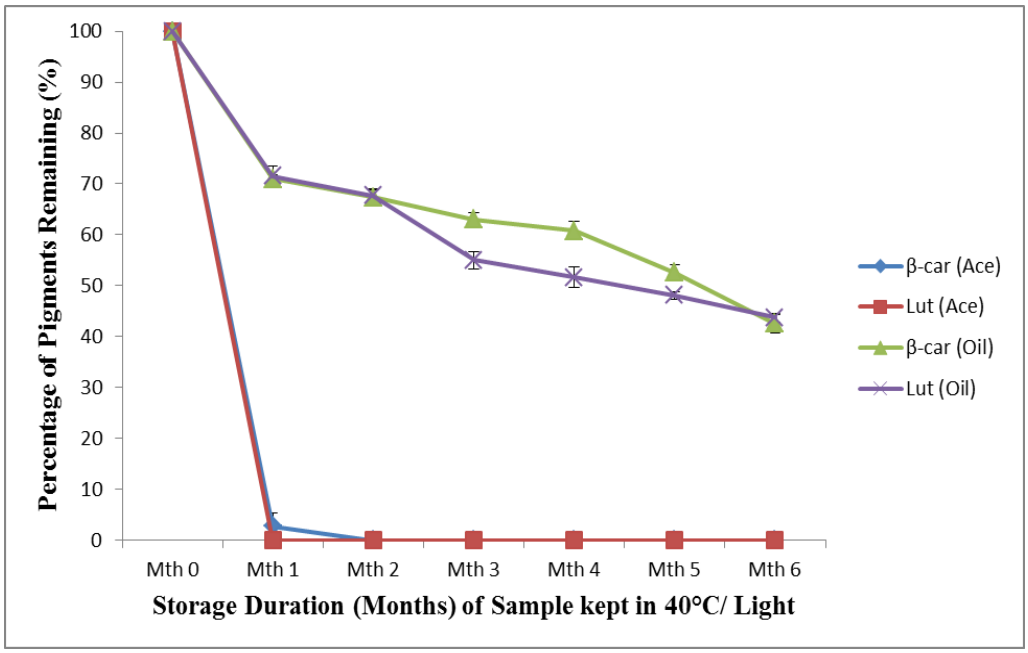


Fig 4.31 Stability of β-carotene and lutein pigments in acetone and soybean oil stored in 40°C in the presence of light (n=3)

4.4 DISCUSSION

Various methods have been proposed for the extraction of carotenoids from multiple sources. In this study, several factors are taken into consideration in the extraction of natural yellow pigments, namely extraction solvents, percentage of potassium hydroxide and duration of saponification process. However, these factors are also influence by the types of plant materials used for extraction.

Generally, carotenoids are pigments that are very soluble in apolar solvents but not in water and are thus known as “lipochromes”. Due to their hydrophobic nature, organic solvents are usually used to extract carotenoids. The types of solvent used in extraction are important in determining the total amount and composition of the extracted product due to their different polarity and affinity to attract different metabolites from the leaf samples. The different types of solvents used often plays a role in the extraction efficiency on different plant tissues and are likely to be dependable on the resistancy of the plant cell to the solvent. In this case, higher extraction efficiency means higher concentration of both β-carotene and lutein pigments extracted from the sample source.The rupture of the plant cells during the grinding process will also facilitate the extraction of pigments from the plant cells. The extraction of pigments using solvents system is also often regarded as a non-equilibrium and unsteady state process (Liu *et al*., 2009).

Several types of solvents were used in previous studies such as acetone, methanol, tetrahydrofuran (THF) and combination of petroleum ether and tetrahydrofuran (4:1). The extraction of β-carotene and lutein pigments is extremely important as the colour of the natural yellow dye depends on the concentrations of these pigments. These two

pigments are the major carotenoids found in leaves although other carotenoids have also been reported in trace amount such as, neoxanthine and violaxanthine (Wills and Rangga, 1996).

In this study, acetone which has the ability to act both as polar and non polar was found to be the best extraction solvent due to the higher intensity of the yellow colour observed in the sample extract as well as higher concentrations of both the β-carotene and lutein pigments recorded (Fig 4.1 and 4.2). Acetone is considered to be polar because it is immiscible with water and non polar due to the presence of carbonyl group in the structure. Besides that, acetone also has the ability to break cells and is easy to be removed via evaporation due to its low boiling point. Methanol, which has been suggested in previous extraction studies, did not function as a good extraction solvent for the *I. batatas* leaves, as observed in the low intensity of the yellow pigments extract and the low concentrations of the β-carotene and lutein pigments obtained (Wang and Liu, 2009). Besides, methanol was also found to be relatively poor in extracting lutein, which contribute significantly to the colour of the natural yellow pigments extract. Although several studies has reported methanol as a powerful extraction solvent, it was documented that their extraction efficiency is not always superior to other solvents when natural samples such as leaves are used (Latasa and Bidigare, 1998).

From previous study, THF was found to be effective in extracting carotenoids from fruit juices (Arena *et al*., 2000). The concentration of β-carotene and lutein pigments extracted using tetrahydrofuran (THF) were ranked second highest behind acetone, but the colour of the extract was light green instead of yellow. This implies the presence of chlorophyll pigments, which was not successfully removed during the saponification process. Tetrahydrofuran is the most polar in the ether family and is a water miscible organic solvent, hence the *I. batatas* leaf extract may not be thoroughly saponifified

when the aqueos potassium hydroxide was added. On the other hand, Bulda *et al.* (2008) reported the mixture of petroleum ether, a non polar solvent and tetrahydrofuran, a water miscible polar solvent (4petroleum ether:1 tetrahydrofuran) to be a good combination to extract the carotenoids pigments from vegetables' seed (Bulda *et al.*, 2008). However, contradicting result was observed for the extraction of *I. batatas* leaves using this solvent combination. Pale yellow coloured extract and low concentration of β-carotene and lutein pigments was obtained from the extraction process.

Saponification is an important step in the carotenoid extraction process to remove chlorophyll pigments from leaf extracts, to remove unwanted lipids and to hydrolized carotenoid esters (Granado *et al*., 2001). Potassium hydroxide (KOH) is a commonly used caustic agents in the saponification process during carotenoids extraction. The duration of hydrolysis, temperature, potassium hydroxide concentration (%) and the number of liquid-liquid partitioning are particularly important to avoid the loss of carotenoid from degradation (Granado *et al*., 2001). The saponification process in this study functioned to remove chlrorophyll from the leaf extract which can be characterised by the formation of a dark oily green ring on the surface of the saponified mixture.

The leaves extract was not completely saponified when 10% potassium hydroxide (KOH) was used and hence extraction cannot be performed on the extract. The leaves extract was not saponified although the incubation period was extended for 6 hours. Therefore, it can be concluded that 10% of aqueous KOH solution was insufficient to saponify the leaves sample. On the other hand, both the β-carotene and lutein concentrations in the extract was found to be significantly lower when 60% KOH was used instead of 40% KOH. The rationale might be the higher concentration of potassium hydroxide (60% KOH) used may facilitate the formation of soap and hence

lowering the level of β-carotene and lutein pigments extracted. Besides, the browning process observed in the leaves extract may be due to oxidation of the phenolics group in the sample. Thus, it can be concluded that 40% of KOH is the better saponification agent to saponify the leaves extract.

Lasellier *et al*. (1993) concluded in their study that saponification process is not essential when chlorophyll levels are low in leaves sample since an aldol condensation to β-8'apocarotenal may occur during the saponification process. However, saponification process in vegetables and leaves is slightly different from other sources since carotenoids are embedded in the complex vegetables matrices. Previously, it was found that saponification process can be completed in 2 min when performed at 100°C, but this comes with drawback of possible isomerisation of the carotenoids in the sample (Craft and Granado, 1993). However, milder saponification conditions have been suggested such as the inclusion of different antioxidants (nitrogen, ascorbic acid or BHT) into the saponification mixture as well as minimization of the exposure to light during saponification, which have shown improvement in the overall carotenoids yield (Kimura *et al*., 1990; Craft and Granado, 1993).

The third factor studied was the duration of the saponification process. In previous studies, Sharpless *et al.* (2004) and Ng and Tan (1988) suggested a 30 min and 4 hours saponification time, whereas Trujillo *et al*. (1990) used overnight saponification to saponify their samples. However, in this study, 2 hours saponification time was included and used to compare with the results from the previously suggested durations. It was found that the different saponification durations shown almost similar yellow-coloured sample and no significance differences were observed in the concentrations of the β-carotene and lutein pigments. However, 30 min and overnight saponification yield slightly lower lutein pigments concentration compared to 2 hours and 4 hours of

saponification. The lower level of lutein observed after overnight saponification might be due to the oxidation of lutein as the result of oxygen exposure. Two hours of saponification duration was chosen for the extraction process due to the higher amount of lutein extracted. Butylated hydroxytoluene (BHT) was also added to the extracted sample to prevent oxidation of the carotenoids in the sample throughout the extraction process (Nonier *et al*., 2004).

Previous studies on the extraction of carotenoids from green leaves extracts employed the usage of chromatography instruments such as HPLC and LC to separate the carotenoids from the chlorophylls. However, these processes are often expensive and requires skilful technicians to conduct (Bulda *et al*., 2008) and hence, liquid-liquid extraction method was used to extract the carotenoids from the leaves in this study. The current source of the natural yellow dye is from *Tagetes erecta* or the African Marigold. Unfortunately, approximately 50% of these pigments are lost during the extraction process due to oxidation and degradation (Barzana *et al*., 2002). Therefore, optimisation of the extraction process is a crucial step to maximise the pigments levels in the product and to minimise loss during processing. Besides, the drying process of the *Tagetes erecta* flowers prior to extraction was also found to contribute to the loss of carotenoids (Barzana *et al*., 2002) and hence fresh *I. batatas* leaves were used throughout this study.

The mean concentrations of β-carotene and lutein obtained in the extraction of *I. batatas* leaves using the optimised conditions were found to be 10.02 mg/100g FW and 10.56 mg/100g FW respectively. Although the *I. batatas* leaves in this study contained lower xanthophylls levels compared to *Terminalia catappa* leaves (24 mg/ 100g), nevertheless, the content was higher compared to *Amaranthus udinis* (12.6 mg/100g), *Mangifera indica* (2.21 mg/100 g), *Ashirantes aspera* (2.67 mg/100 g) and *Carica*

papaya (2.7mg/ 100 g) (Bhaskarachary *et al*., 1995; Lopez-Hernandez *et al*., 2001). Hence, this makes the *I. batatas* leaves a cheap and abundant source of natural yellow pigments.

The double bonds structure of the carotenoids makes them vulnerable to degradation upon exposure to oxygen, light, high temperature and acid (Chen and Chen, 1993; Henry *et al*., 1998). Carotenoids degradation involves the changes of the double bond from trans to cis configuration (Britton *et al*., 1995). The degradation pattern of carotenoids are also influenced by factors such as reaction medium, temperature, physical state, types of pigments and environmental conditions (Pesek and Warthesen, 1988; Minguez-Mosquera and Jaren-Galan, 1995). Lipoxygenases was also found to accelerate the oxidation reactions of the samples and eventually lead to pigments degradation (Biascs *et al*., 1992; Gordon and An, 1995).

The disintegration of carotenoids during the degradation process always starts in the open end of the molecule before reaching the β-ionone ring. This causes the double bonds to be saturated and the characteristic colour of the carotenoids to disappear. Previous studies on the oxidative stability of three carotenoids (lycopene, β-carotene and lutein) in an oil based model system shown that lutein exhibited the highest stability among the different carotenoids. However, these results cannot be compared with the complex food systems that are often incorporated with numerous components such as proteins, carbohydrates and lipids, which have protective functions (Henry *et al*., 1998). Previous studies have also reported the stability of pigments stored in the presence of light, however pigments stability at high temperatures are not documented in literature (Lopez-Hernandez *et al*., 2001).

An earlier study had suggested that β-carotene has the ability to protect soybean oil against photo-oxidation due to their high number of conjugated bonds compared to the other carotenoids (Yen and Chen, 1995). In contrast, a study conducted by Jia *et al*. (2007) concluded that the addition of soybean oil to the β-carotene pigments has been shown to increase the β-carotene stability (Jia *et al*., 2007). Hence, β-carotene and soybean oil can act interchangeably to protect each other from the oxidation process. Studies also showed that extraction of carotenoids into the oil could improve their efficacy compared to their natural occurrence in vegetables (van Lieshout *et al*., 2001). Besides that, the fatty acid composition as well as the types and levels of antioxidant compounds may also contribute to the stability of the carotenoids in the sample (Porter *et al*., 1995).

In this study, the stability of the yellow pigments extract from *I. batatas* was investigated in two different matrix systems, namely in acetone and soybean oil. In previous study conducted by Ceron-Garcia *et al*. (2010), acetone and olive oil were used to study the stability of the carotenoids extracted from *Scenedesmus almeriensis*. However, soybean oil was used in this study instead of olive oil so that the colour changes of the extract can be observed visually throughout the storage duration due to the lighter colour shade of the soybean oil. Besides, soybean oil was also used extensively worldwide due to its wide availability and economical value. Soybean oil accounted up to 75% of the total vegetable oil consumption in the United States with a total production exceeding ten folds compared to cottonseed oil production (Weiss, 1983). In addition, its low saturated fat and no-cholesterol features make it a suitable and healthy choice to store the extracted yellow pigments.

Scanning spectrophotometer was used to detect the presence of pigments (chlorophylls, carotenoids and anthocyanin) in the leaves extract between 380 nm to 700 nm wavelength. The characteristic three peaks in the chromatogram is a major indicator of the presence of carotenoids in the extract. The carotenoids in acetone shown absorption peaks at 432 nm, 446 nm and 473 nm while formation of peaks at 434 nm, 448 nm and 475 nm were observed in soybean oil. A shift of 2 nm was observed in the peaks, which may be contributed by the different medium used that causes changes in light refraction. The peaks observed in this study were found to be a combination of the all-trans β-carotene and all-trans lutein as observed in the study by Tai and Chen (2000). Absorbance reading via scanning spectrophotometer showed that the yellow extract did not contained traces of chlorophyll, a problem often associated with extraction of carotenoids from plants especially leaves. This is because, chlorophyll *a* are usually detected at 680nm while chlorophyll *b* are detected at 650nm wavelength (Lichtenthaler, 1987).

Results showed that in short-term stability study, increase in temperature brought greater instability to lutein pigments compared to β-carotene pigments. However, this result was in contrast with the study conducted on Daylily (*Hemerocallis disticha*) flowers, which all-trans β-carotene was found to degrade more rapidly compared to all-trans lutein when subjected to hot-aired drying (Tai and Chen, 2000). In addition, short-term storage of lutein pigments in the presence of light also showed higher pigments loss compared to when kept in higher temperatures. This finding was found to be contradicting with the results from the β-carotene pigments kept in acetone in which temperature serve to contribute more to pigment loss. In the long-term stability study, the lutein pigments in soybean oil were found to attain higher stability in -20°C compared to β-carotene regardless of the matrix systems. Besides that, both short-term

and long-term storage also shows that β-carotene pigments were less stable in -20°C compared to 4°C. Degradation of pigments in this case was mostly contributed by the presence of oxygen since photo-oxidation does not occur in the absence of light.

Besides that, photo-oxidation process also occur in the presence of light which explains why samples kept at the same temperature showed more extreme degradation pattern in the presence of light. Long-term storage at 25°C also showed that β-carotene pigments had lower stability compared to the lutein pigments. A greater loss in pigments concentration was observed at a higher storage temperature (40°C) especially during the first month of storage compare to when the samples were kept at 25°C. From this study, it can be concluded that the degradation of pigments in the natural yellow pigments extract can be affected by the different storage matrices used. The stability of the samples in soybean oil was better maintained compared to when kept in acetone. Acetone was not a good matrix system for storage because the pigments can be degraded easily at the presence of light, oxygen and high temperatures. Soybean oil on the other hand, works better to maintain the stability of the pigments stored both in the dark and under light conditions.

A study conducted by Lin and Chen (2005) showed that the concentration of lutein pigments in tomato juice decreased more rapidly with the increase in storage temperatures and in the presence of light. Complete degradation of lutein in tomato juice kept in dark at 35°C was achieved upon 4 weeks of storage while in the presence of light, complete degradation was observed only after 3 weeks of storage. Besides, it was also found that β-carotene pigments were more stable compared to lutein pigments in the tomato juice sample. In a separate study, lutein pigments extracted from *Scenesmus almeriensis* and kept in olive oil extract showed high stability with approximately 80% of recovery after storage for 6 months at -18°C (Ceron-Garcia *et al*.,

2010). Comparatively, in this study, the lutein pigments from *I. batatas* leaves showed approximately 90% recovery after 6 months of storage at -20°C in soybean oil. Thus, this showed that soybean oil is a suitable medium to store carotenoids pigments. Besides that, a similar pattern was observed in *Scenesmus almeriensis* with a steady decrease in lutein pigments in acetone, which recorded 40% pigments loss in six weeks duration when kept at 25°C. These findings concluded that an oil based system is indeed better in maintaining the stability of the carotenoid pigments compared to acetone.

During storage, both the isomerization and degradation process of the pigments might occur simultaneously which often resulted in the changes of the pigments concentration (Pesek *et al*., 1990). The oxidation process that occur leads to the formation of highly reactive species such as alkyl and peroxyl radicals and these radicals will increase the degradation of easily oxidizable compounds, such as lutein (Krinsky and Yeum, 2003).

As a conclusion, the optimum conditions for the extraction of the natural yellow pigments from *I. batatas* leaves was achieved using acetone as extraction solvents, 40% potassium hydroxide (KOH) and a saponification duration of 2 hours. The soybean oil matrix was selected as a better storage medium in maintaining the stability of the β-carotene and lutein pigments in the sample compared to acetone. Higher temperature and presence of light also brought greater instability to the pigments in the sample. Besides, from this study, illumination plays a more determinant role in the stability of pigments compared to temperature.

CHAPTER 5

Qualitative Analysis and Antioxidant Activity of Yellow Extract from *Ipomoea batatas* leaves

5.1 INTRODUCTION

Qualitative analysis is used to analyse and determine the chemical compositions of a substrate. Originally, the separation of carotenoids was performed using open column chromatography but this method is no longer used since it requires large amounts of sample (Almeida and Penteado, 1988; Su *et al*., 2002). Since the development of technology such as the high-performance liquid chromatography (HPLC), both the normal and reversed-phased methods have been extensively used to separate the two main groups of carotenoids, which are the xanthophylls and carotenes. Coupled with the usage of HPLC are usually the ultraviolet-visible (UV-Vis) detector and the newer photodiode array detector (DAD) which allow data to be collected continuously during analysis (Huck *et al*., 2000). Mass spectrophotometry / mass-spectrometry (MS/MS) detector is often used together with the liquid chromatography method to identify complex matrices, due to its higher sensitivity compared to a DAD (Feruzzi *et al*., 1998; Rozzi *et al*., 2002).

The presence of pesticides in sample extract is a common problem faced when plants are used as extraction source. Generally, pesticides are a group of diversified chemical compounds and include insecticides, fungicides, herbicides and rodenticides. While the usage of these chemicals have vastly contributed to higher crop yield and limit the spread of harmful crop diseases, their usage has also brought adverse effects to human health and well-being. Pesticides are used in *I. batatas* farming to ensure the growth of

healthy leaves and roots. Currently, more than 100000 agricultural food samples are analysed each year for pesticide residues. The absence of pesticides in the final product is often the ultimate goal especially in the food industry.

Natural antioxidants have been isolated and studied from various plants including cereal crops, vegetables, leaves, roots, herbs and spices (Shon *et al*., 2003; Wettasinghe and Shahidi, 1999). Antioxidants have the ability to scavenge free radicals and protect the body from potential damages from these free radicals. Numerous groups of phytochemicals, such as carotenoids, ascorbic acid, α-tocopherol and polyphenols contributed to the antioxidant properties in vegetables (Salah *et al*., 1995; Edge *et al*., 1997; Papas, 2002). Furthermore, antioxidants ability to prevent food deterioration and to extend shelf life is another reason why it is widely used in the food industry (Hotta *et al*., 2002). Hence, the presence of antioxidants in food products will be a value added benefit to the products.

There are basically three main functions of antioxidants in the human body. Firstly, they function to suppress the formation of free radicals and reactive oxygen species in human body. This will reduce the harmful effects caused by these free radicals. Secondly, antioxidants act as scavenger, hunting down the available free radicals in the body. Thirdly, they act as substrate for the free radicals and reactive oxygen species in human body (Wu and Simin, 1999). The examples of natural antioxidants that can be acquired through diet are chlorophylls, flavonoids, vitamin C, selenium and lycopene.

Natural antioxidants in wine, fruits and vegetables have been studied widely due to their benefits and commercial values. However, in many fruits and vegetables, antioxidant capacity was found to be contributed by non-vitamin C phytochemicals (Wang *et al*., 1997). Extraction often resulted in the reduction of antioxidant properties in the final

extract due to the long processing time and exposure to oxidative agents such as high temperatures, oxygen and other factors. In this chapter, the effect of the extraction process on the antioxidant properties of the yellow extract will be studied by comparing the antioxidants contents and antioxidant activities of the leaf and carotenoids extracts from *I. batatas* leaves.

The objectives of this study are to determine the composition of the yellow extract from the *I. batatas* var. Oren leaves using LCMS/MS, to determine the presence of pesticides residues in the yellow pigments extract, to determine the effects of extraction process on the total phenolics and total flavonoids content in the yellow extract via Folin-Ciocalteau assay and Vanillin-HCl assay respectively and finally, to determine the radical scavenging activity and reducing power capabilities of the yellow pigments extract via DPPH radical scavenging assay and reducing power assay.

5.2 MATERIALS AND METHODS

5.2.1 Qualitative analysis

5.2.1.1 Lutein and β-carotene Standards

The stock for β-carotene standard was prepared by dissolving 100 mg of β-carotene standard (CalBioChem, Merck) in 5 ml of hexane:acetonitrile (1:1). Then, 0.1 ml of the standard's was diluted with 1.9 ml of hexane:acetonitrile (1:1) to a concentration of 1000 mg/L. This is followed by the dilution to a final concentration of 10 mg/L (equivalent to 10 ppm) with acetonitrile:methanol (4:6). The standard was filtered using a syringe filter (Whatman) with pore size 0.45 µm before analysis.

Lutein standard was prepared by dissolving 1 mg of lutein (Sigma) standard in 0.5 ml of hexane to make a concentration of 2000 mg/L. The standard (0.1 ml) was then added with 0.9 ml of hexane:acetone (1:9) followed by dilution with acetonitrile:methanol (4:6) to a final concentration of 2 ppm. The lutein standard was filtered using a syringe filter (Whatman) with pore size 0.45 µm before analysis.

5.2.1.2 Sample Preparation

Ipomoea batatas var. Oren leaves was ground to powder form using pestle and mortar and 0.5 g was extracted with 50 ml of acetone. The mixture was shaken overnight in an orbital shaker at room temperature (Stuart Scientific). The extracted sample was filtered with Whatman No.1 filter paper into a 50 ml Falcon tube and added with 0.1g of butylated hydroxytoluene (BHT). The leaves sample was re-extracted with 20 ml of acetone following the same protocol as mentioned above. Tubes containing the filtrate were placed in ice and in the dark to avoid pigments degradation.

The filtrates were placed in an evaporatory flask and the solvent was removed under vacuum using a Butchi rotary evaporator. Then, 20 ml of 40% aqueous potassium hydroxide (KOH) was added into the extract and the mixture was left to saponify at 45ºC. Acetone (25 ml) was added to the saponified sample at 50ºC and the mixture was poured into the separating funnel. The bottom layer was discarded while the upper layer was collected in an evaporatory flask. The extract was evaporated using a rotary evaporator leaving a layer of oleoresin.

The oleoresin layer was then added with 10 ml of petroleum ether:tetrahydrofuran (4:1) and a layer of green lipid will immediately formed at the base of the tube. The upper yellow layer was used for subsequent qualitative analysis. Besides that, the β-carotene and lutein pigments in the yellow extract were also quantified using the spectrophotometric method (Bulda *et al.*, 2008). For storage purposes, 1 ml of yellow pigments extract was dried under a stream of flowing oxygen-free-nitrogen (OFN) gas and was kept in -80°C freezer until analysis. All the experiments were performed in triplicates and analysis was performed within a week after extraction.

5.2.1.3 Determination of Carotenoids using Liquid Chromatography Mass Spectrometry/ Mass Spectrometry (LCMS/MS)

The carotenoids composition in the sample extract was detected using a LCMS/MS system (Applied Biosystems 3200Q Trap LCMS/MS). Shimadzu Ultra Performance Liquid Chromatography system was used for the separation of carotenoids while identification was done using a mass spectrometry. Separation was achieved using a C_{18} column (Phenomenex Aqua, 50mm x 2.0mm x 5µM) and the buffer methanol:acetonitrile (40:60) was added with 0.1% of formic acid and 0.1% of ammonium formate. Analysis was performed at 0.5 mL/min flow rate under isocratic run with (Multiple Reaction Monitoring) MRM run time of 5 min. The β-carotene and lutein standards were analysed for their MRM transition values, which will be used to determine the presence of β-carotene and lutein in the yellow pigments extract.

5.2.1.4 General Screening for Active Carotenoids

The yellow extract was also screened for active carotenoids using LCMS/MS system (Applied Biosystems 3200Q Trap LCMS/MS) based on the method described in section 5.2.1.3. The analysis was conducted using electrospray mass spectrometry (EMS) and the data was collected using the MS/MS system. Two different ionisation modes (positive and negative) were used for scanning. The mass spectrum obtained from the analysis was used to identify the compound by comparing the mass-to-charge (m/z) ratio values with the database provided by the National Institute of Standards and Technology accessed at http://webbook.nist.gov/.

5.2.2 Pesticide Analysis on Yellow Pigments Extract

The yellow pigments extract was subjected to pesticides screening for the detection of organophosphorus insecticides, organochlorinated insecticides and herbicides residues using gas chromatography-mass spectrometry (GCMS) method. The sample was sent to Consolidated Laboratory (M) Sdn. Bhd. for analyses. The lists of herbicides and insecticides screened in the analyses are attached in Appendix 1.

5.2.3 Antioxidant Analysis on the Yellow Pigments Extract from *Ipomoea batatas*

5.2.3.1 Sample Preparation

Firstly, the *Ipomoea batatas* var. Oren leaves were ground into fine powder using a mortar and pestle in liquid nitrogen. Leaf extract was prepared by adding methanol to the leaves sample, followed by filtration and the supernatant were used for antioxidant analyses.

The yellow extract used for analysis was prepared using the method described in section 5.2.1.2. The yellow extract was dissolved in petroleum ether:tetrahydrofuran (4:1) and was quantified based on the equations by Bulda *et al* (2008). Then, the yellow extract was dried using a rotary evaporator (Butchi) and dissolved in 15 ml of methanol prior to analysis.

5.2.3.2 Total Polyphenols (Folin-Ciocalteau) Assay

Sodium Carbonate

Anhydrous sodium carbonate (BDH) (8 g) was dissolved in 32 ml of distilled water and the mixture was brought to boil using a hot plate. After cooling, the mixture was left overnight at room temperature and was filtered through Whatman No.1 filter paper. The volume of the mixture was made up to 40 ml with distilled water.

Preparation of Gallic Acid Standard

Stock solution was prepared by adding 0.1 g of gallic acid (Sigma) to 2 ml of methanol and the volume was made up to 20 ml with distilled water. The different concentrations of the gallic acid standards were prepared according to Table 5.1 and the final volume was made up to 10 ml with distilled water.

Table 5.1 Gallic Acid Standard Preparation

Concentration (mg/L)	100	200	300	400	500
Volume of stock solution (ml)	0.2	0.4	0.6	0.8	1.0

Analysis

Total polyphenols content was measured using the folin-ciocalteau assay based on the protocols by Waterhouse (2002). Gallic acid standard (50 µl) of different concentrations was added with 3.95 ml of deionised water followed by 250 µl of folin-ciocalteau reagent (Sigma) before the mixture was incubated for 5 min. Sodium carbonate solution (750 µl) was then added and the mixture was incubated for 2 hours at room temperatures. Absorbance was recorded at 765 nm and blanked with distilled water.

5.2.3.3 Vanillin-HCl Assay

Reagent A: 8% Hydrochloric acid (HCl) in methanol

Eight millilitres of concentrated hydrochloric acid (HCl) was added to 90 ml of methanol (Merck). The volume of the mixture was then made up to 100 ml with methanol.

Reagent B: 1% Vanillin in methanol

One gram of vanillin (Sigma) was dissolved and made up to 100 ml volume with methanol (Merck).

Vanillin-HCl solution

The vanillin-HCl solution was prepared by mixing 50 ml of Reagent A with 50 ml of Reagent B.

Preparation of catechin standard

Catechin (Sigma) was dissolved into methanol to a final concentration of 1 mg/ml as a stock. The different concentrations of the catechin standard were prepared according to Table 5.2 and the final volume was made up to 10 ml with methanol.

Table 5.2 Catechin Standard Preparation

Concentration (µg/ml)	20	40	60	80	100
Volume of stock solution (ml)	0.2	0.4	0.6	0.8	1.0

Analysis

The vanillin-HCl assay was conducted based on the protocols by Sun *et al*. (1998). Catechin standard (250 µl) was added to 1 ml of vanillin-HCl reagent in a 2 ml microcentrifuge tube. The tube was inverted a few times and was incubated in the water bath for 20 min at 30ºC. Absorbance was measure at 500 nm and blanked with 80% methanol. Analysis was conducted by substituting catechin standard with the leaf/ yellow extract.

The absorbance (A) values for the different concentrations were calculated by the using the following formula:

A_0 = 250µl methanol + 500µl methanol + 500µl 8% HCl

A_b = 250µl methanol + 500µl 1% Vanillin + 500µl 8% HCl

A_c = 250 µl Catechin / sample + 500µl methanol + 500µl 8% HCl

A_s = 250µl Catechin / sample + 500µl 1% Vanillin + 500µl 8% HCl

$A = (A_s - A_b) – (A_c - A_0)$

A calibration curve was drawn based on the A values.

5.2.3.4 DPPH Radical Scavenging Assay

80% Methanol

Eighty millilitres of methanol (Merck) was added with 10 ml of distilled water and the final volume was made up to 100 ml.

0.1 mM of DPPH solution

1,1-diphenyl-2-picryl hydrazyl (DPPH) (Sigma) (39.4 mg) was dissolved in 90 ml of 80 % methanol and the final volume of the mixture was made up to 100 ml.

Preparation of Vitamin C Standard

One hundred milligram of L-ascorbic acid (Sigma) was dissolved in 9 ml of methanol (Merck) and the volume was made up to 10 ml as stock. The different concentrations of the vitamin C standard were prepared from the stock as shown in Table 5.3 and the final volume was made up to 10 ml with methanol.

Table 5.3 Vitamin C Standard Preparation

Concentration (µg/ml)	200	400	600	800	1000
Volume of stock solution (ml)	0.2	0.4	0.6	0.8	1.0

Analysis

DPPH radical scavenging assay was performed based on protocols by Oyaizu (1986) and Bae and Suh (2007). Vitamin C standard (L-ascorbic, Sigma) (500 µl) was added with 1ml of 0.1 mM DPPH solution in a 1.5 ml microcentrifuge tube. The mixture was vortexed for 15 seconds and incubated at 37ºC for 30 min. The absorbance was read at 517 nm and results were expressed in percentage of inhibition of the radicals. Analysis was conducted by substituting vitamin C standard with the extracts. The formula for the calculation is:

$$\% \text{ Inhibition} = \frac{\text{Blank abs at 517 nm} - \text{Final abs at 517 nm}}{\text{Blank abs at 517 nm}} \times 100$$

5.2.3.5 Reducing Power Assay

0.2 M Phosphate Buffer (pH 6.6)

Reagent A

Sodium phosphate monobasic ($NaH_2PO_4H_2O$) (Sigma) (4.82 g) was dissolved in 200 ml of distilled water and the final volume was made up to 250 ml.

Reagent B

Sodium phosphate dibasic heptahydrate ($Na_2HPO_4.7H_2O$) (Sigma) (4.04 g) was dissolved in 200 ml of distilled water and the final volume was made up to 250 ml.

The 0.2 M phosphate buffer was prepared by mixing 205.5 ml of reagent A with 94.5 ml of reagent B.

1% Potassium ferricyanide

One gram of potassium ferricyanide (Sigma) was dissolved in 90 ml of distilled water and the final volume was made up to 100 ml.

0.1% Ferric chloride

Ferric chloride (Sigma) (0.1 g) was dissolved in 90 ml of distilled water and made up to 100 ml.

10% Trichloroacetic acid

Ten gram of trichloroacetic acid (Fisher) was dissolved in 90 ml of distilled water and the final volume was made up to 100 ml.

Preparation of Butylhydroxyanisole (BHA) Standard

One hundred milligram of butylhydroxyanisole (BHA) (Sigma) was dissolved in 90 ml of methanol (Merck) and the volume was made up to 100 ml as stock. The different concentrations of BHA standard were prepared from the stock as shown in Table 5.4 and the final volume was made up to 10 ml with methanol.

Table 5.4 Butylhydroxyanisole (BHA) Standard Preparation

Concentration (µg/ml)	200	400	600	800	1000
Volume of stock solution (ml)	2	4	6	8	10

Analysis

Reducing power assay was conducted based on the protocols by Oyaizu (1986) but with modifications. The extracted sample (50 µl) was added with 200 µl of 0.2 M phosphate buffer and 200 µl of 1% potassium ferricyanide in a 2 ml microcentrifuge tube. The tubes were inverted a few times and incubated for 20 min at 50ºC. Then, 250 µl of trichloroacetic acid was added to the mixture followed by centrifuging at 1000 rpm for 10 min at room temperature. Five hundred microliters of the supernatant was added to 500 µl of distilled water and 100 µl of 0.1% ferric chloride and the mixture was incubated at 37ºC for 10 min. The absorbance for the mixture was read at 700 nm.

5.3 Results

5.3.1 Qualitative Analysis of Yellow Pigments Extract

A single peak was obtained for the β-carotene standard analysed using the LCMS/MS system (Figure 5.1) with an atomic mass units (amu) value of 537.5 and retention time of 2.46 min. The β-carotene pigment in the yellow extract was confirmed with the presence of a single peak at 537.5 amu and at retention time of 2.45 min (Figure 5.2). The area at the bottom of the peak was used to calculate the concentration of the β-carotene pigments in the yellow extract. From the calculation, the β-carotene concentration was found to be 5.4 ppm or 5.4 mg/L and the final concentration of β-carotene in the extract was 10.8 mg/100g FW.

On the other hand, a single peak (Figure 5.3) was observed for the lutein standard at m/z value 569.5 amu and 0.5 min retention time. A single peak with similar m/z value and retention time was observed for the yellow extract and thus confirmed the presence of lutein. The concentration of lutein in the yellow extract was determined from the area under the peak in the chromatograms (Figure 5.3 and 5.4). The concentration of lutein pigments in the yellow extract obtained was approximately 5.0 mg/ml or 5.0 ppm and this accounted to 10.0 mg/100g when converted in terms of mass.

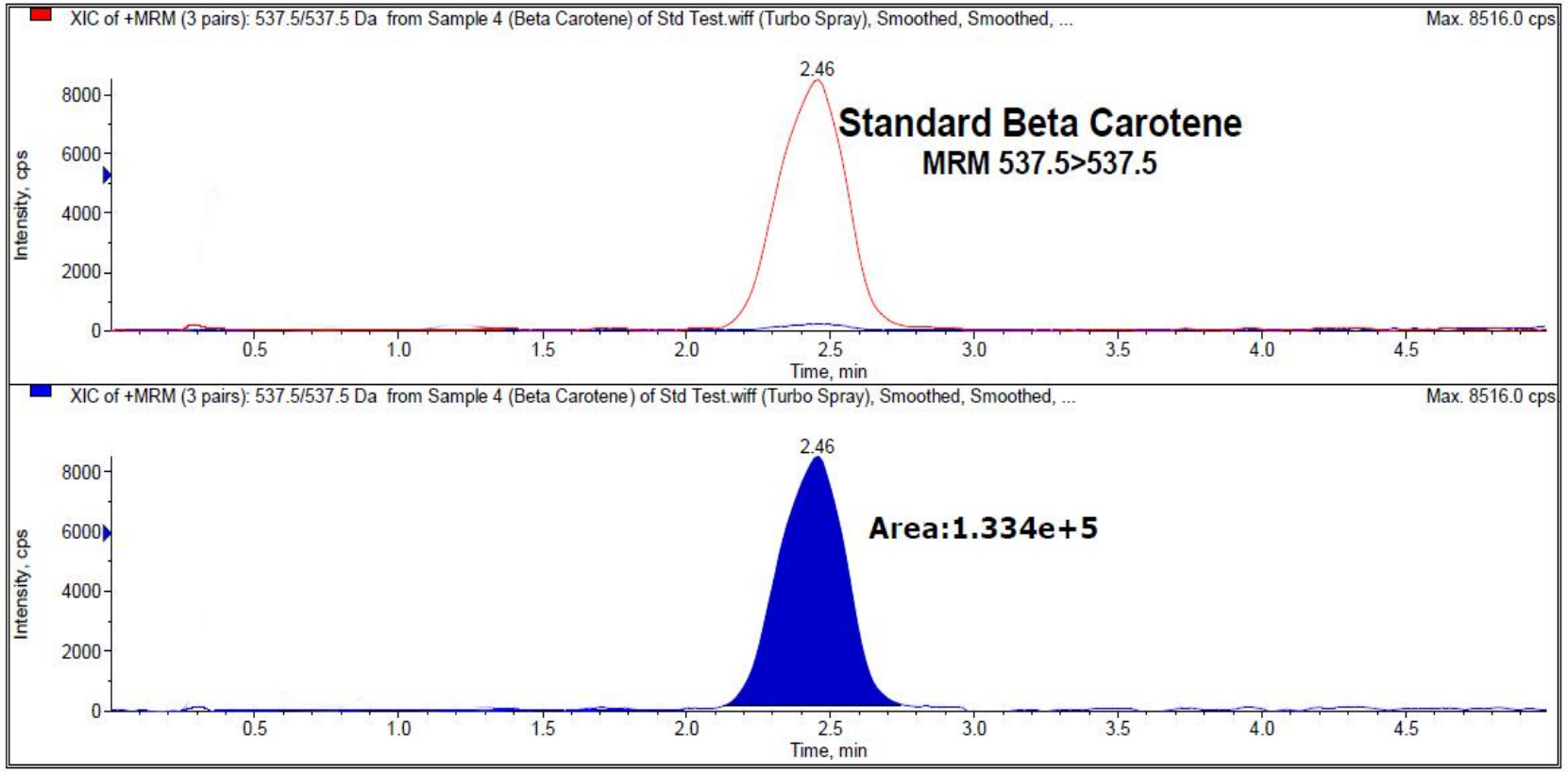


Fig 5.1 Detection of β-carotene standard peak using LCMS/MS

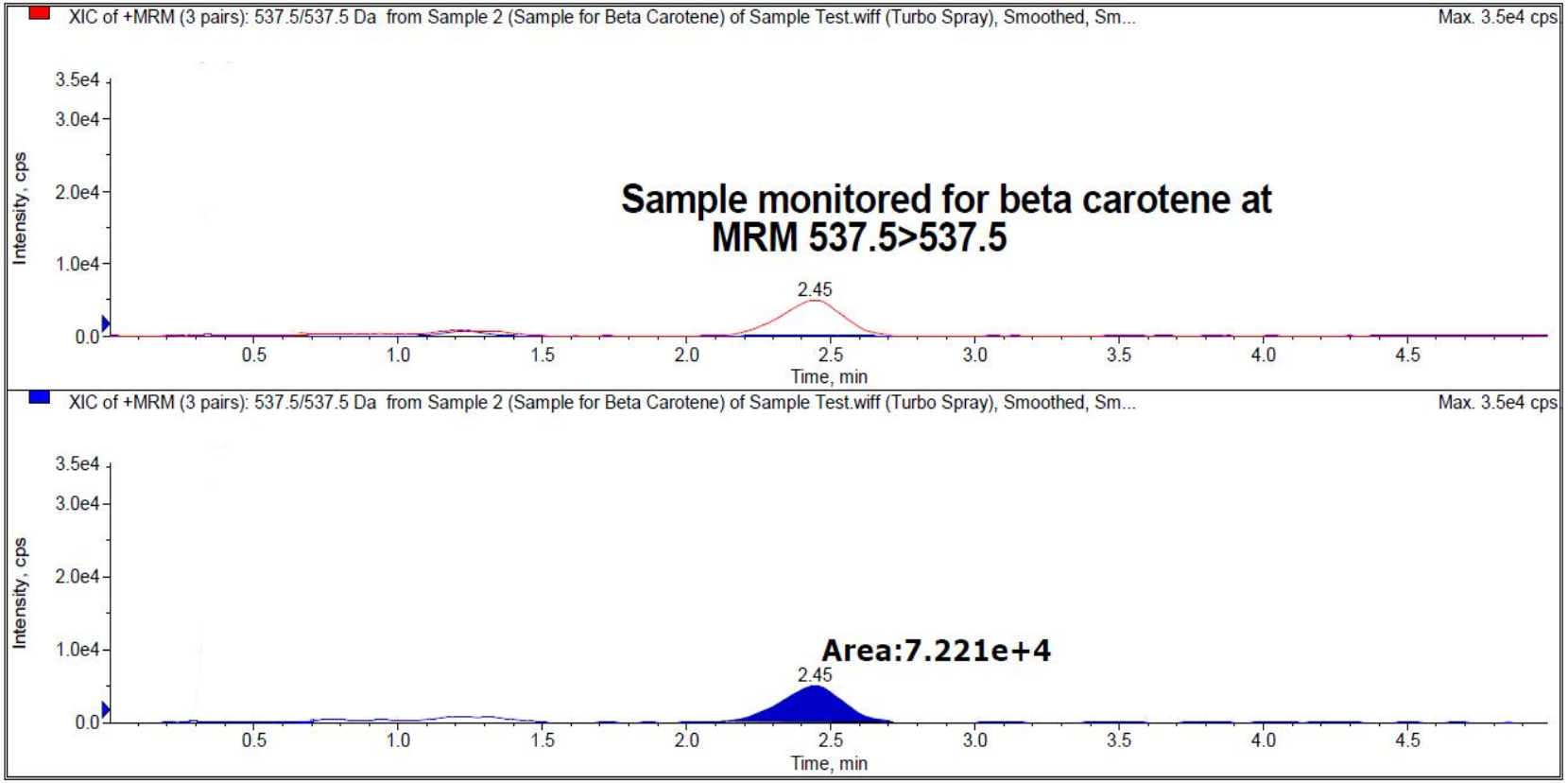


Fig 5.2 Detection of β-carotene peak in the yellow extract from *Ipomoea batatas* leaves using LCMS/MS

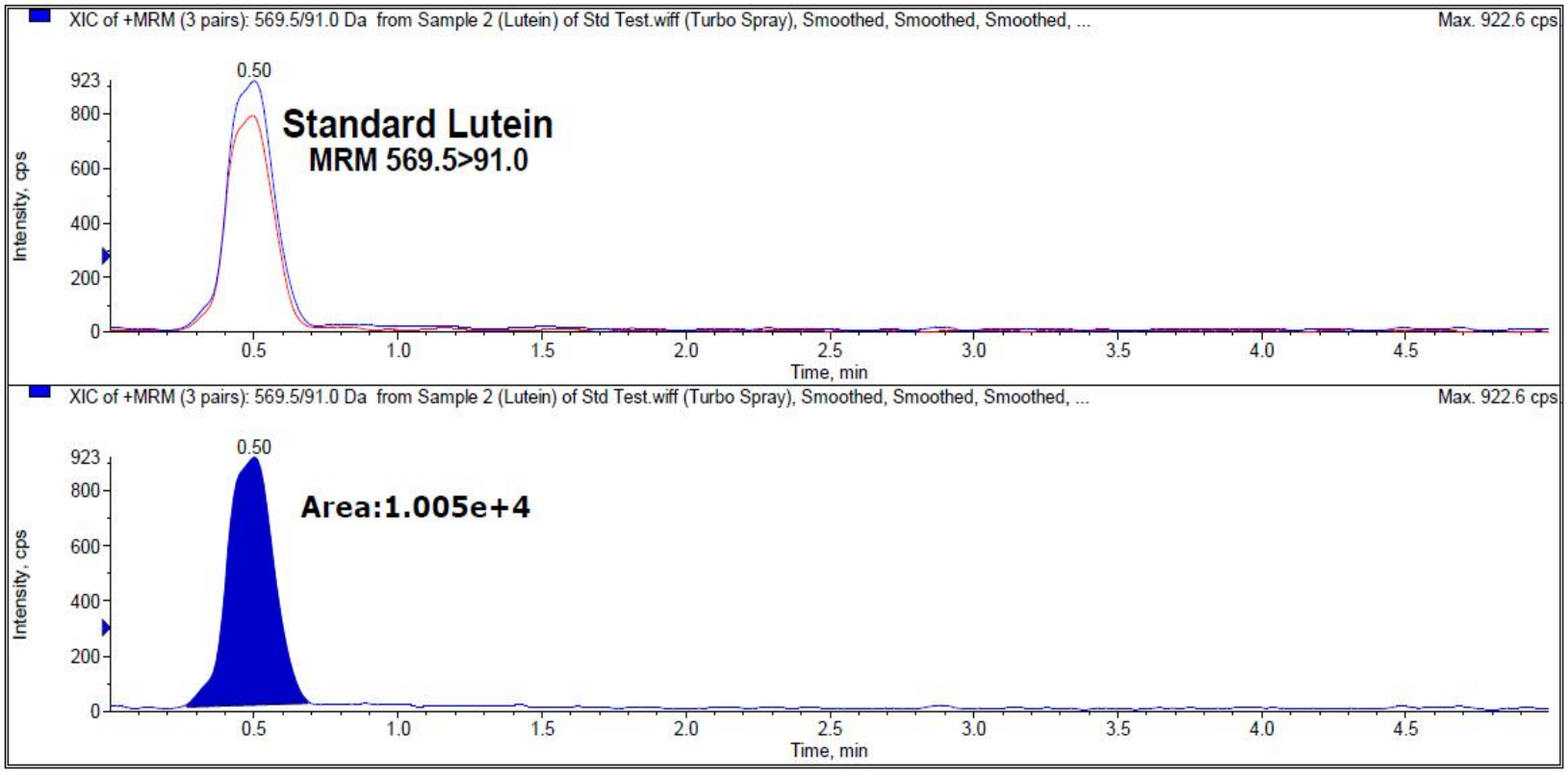


Fig 5.3 Detection of lutein standard peak using LCMS/MS

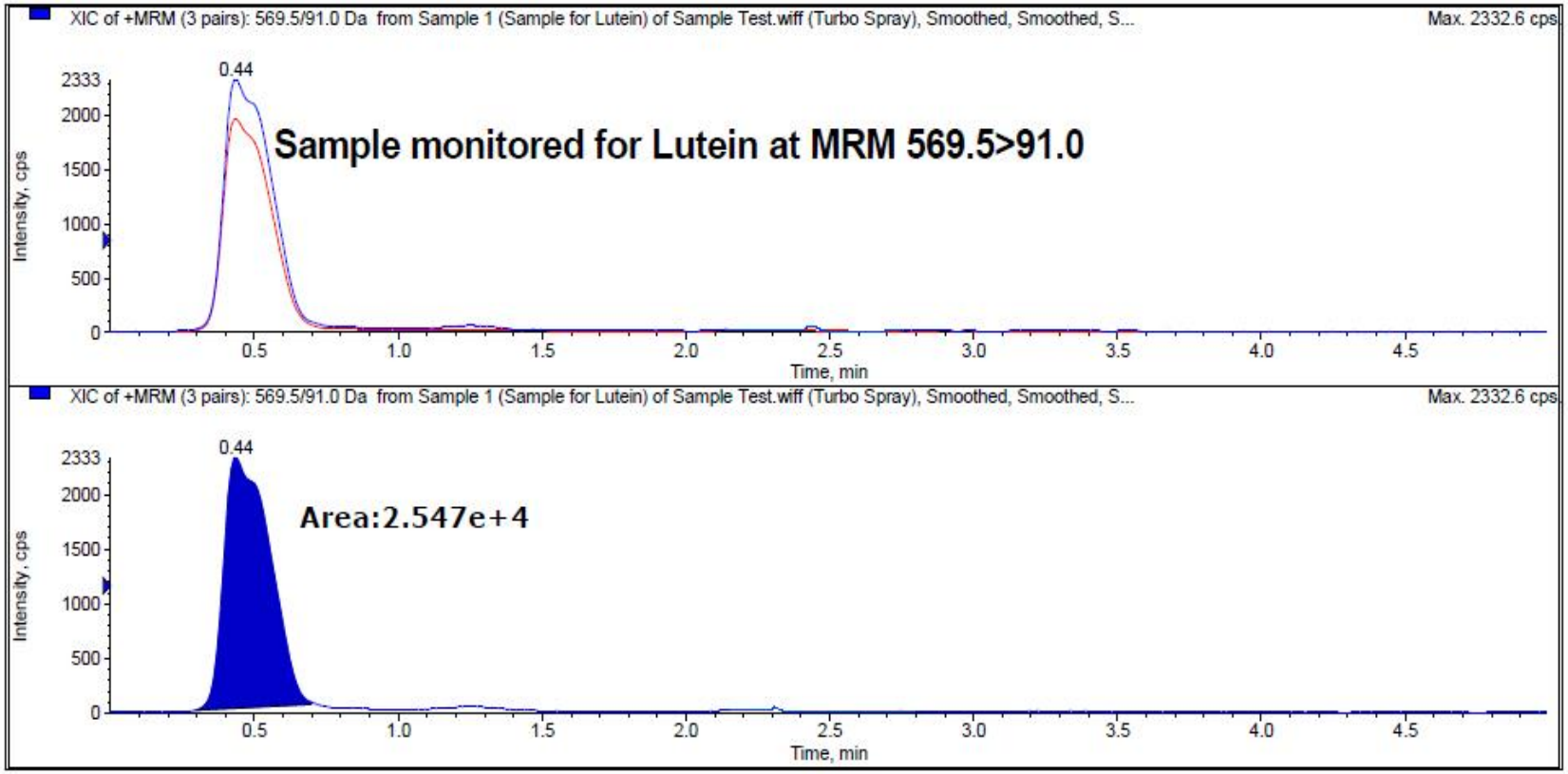


Fig 5.4 Detection of lutein peak in the yellow extract from *Ipomoea batatas* leaves using LCMS/MS

The chromatogram for the yellow extract analysed using the LCMS/MS (positive ionisation mode) is shown in Figure 5.5. Analysis conducted in the negative ionisation mode did not yield any result. It was observed that the yellow extract consist of other compounds besides the β-carotene and lutein pigments. The highest peak detected was the 2-monolinoleoylglycerol trimethylsilyl ether, followed by malonic acid, decanoic acid and fumaric acid, which are the commonly found organic acids in plants. Both the lutein and β-carotene pigments were found in a high concentration in the extract, which may explain the yellow colour observed in the sample. Besides that, beta cryptoxanthin and 4-ketozeaxanthin (an isomer lutein) was also detected in the extracted sample. The mass spectra of some of the compounds in the yellow extract were identified and the structures of these compounds were included with the chromatogram. The results of the analysis can be summarised in Table 5.5.

Table 5.5 Compounds Detected Using LCMS/MS

Compounds	Highest fragment mass (m/z) value	Figure
Lutein	568.5	Fig 5.6
β-carotene	536.5	Fig 5.7
Beta cryptoxanthin	552.5	Fig 5.8
Fumaric acid	526.6	Fig 5.9
Phytofluene	542.6	Fig 5.10
4-ketozeaxanthin	582.7	Fig 5.11
1-monolinoleoylglycerol trimethysilyl ether	498.4	Fig 5.12
Malonic acid	532.4	Fig 5.13
Decanoic acid	554.6	Fig 5.14

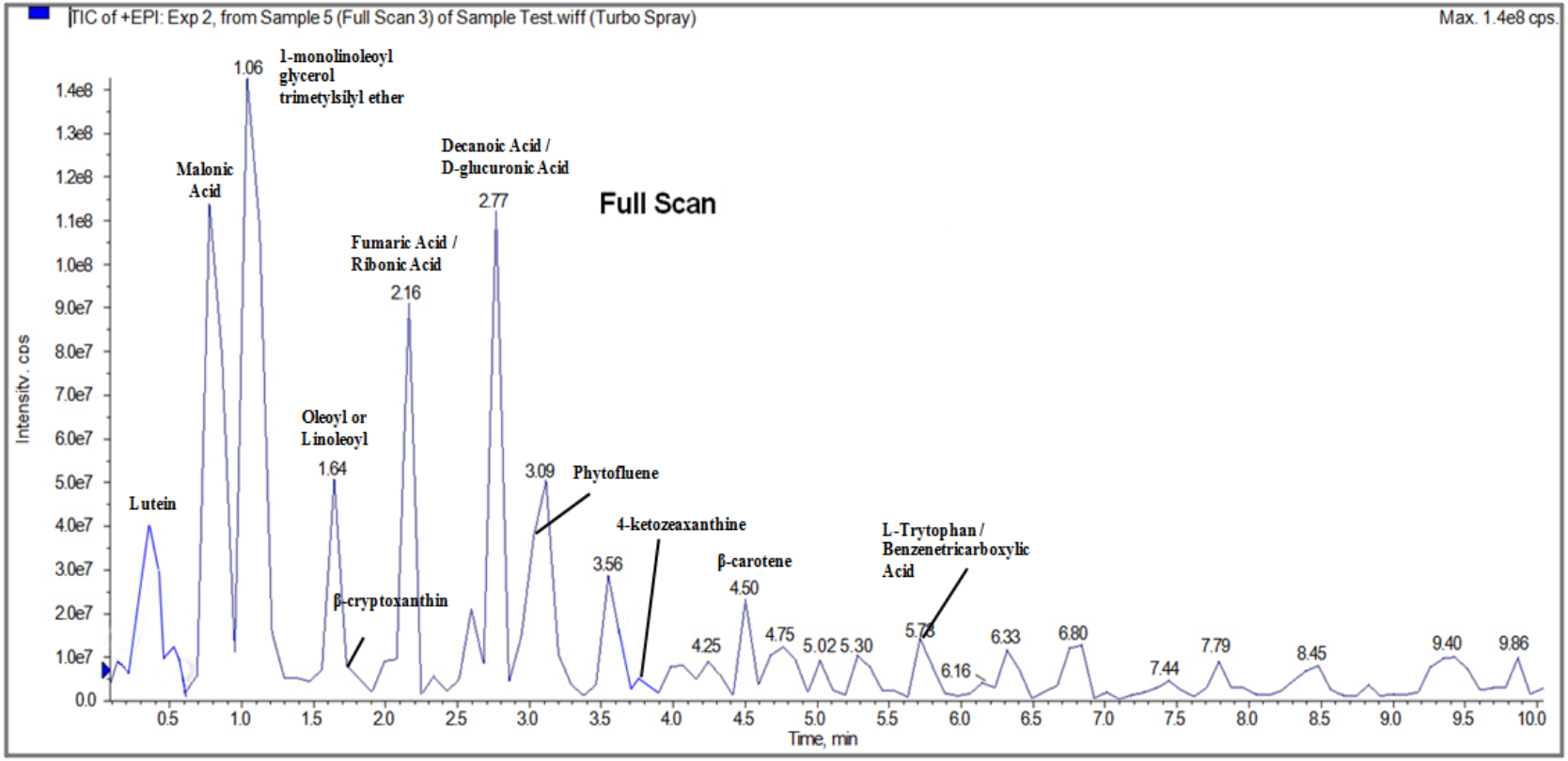


Fig 5.5 Chromatogram of the yellow extract from *Ipomoea batatas* leaves detected using LCMS/MS

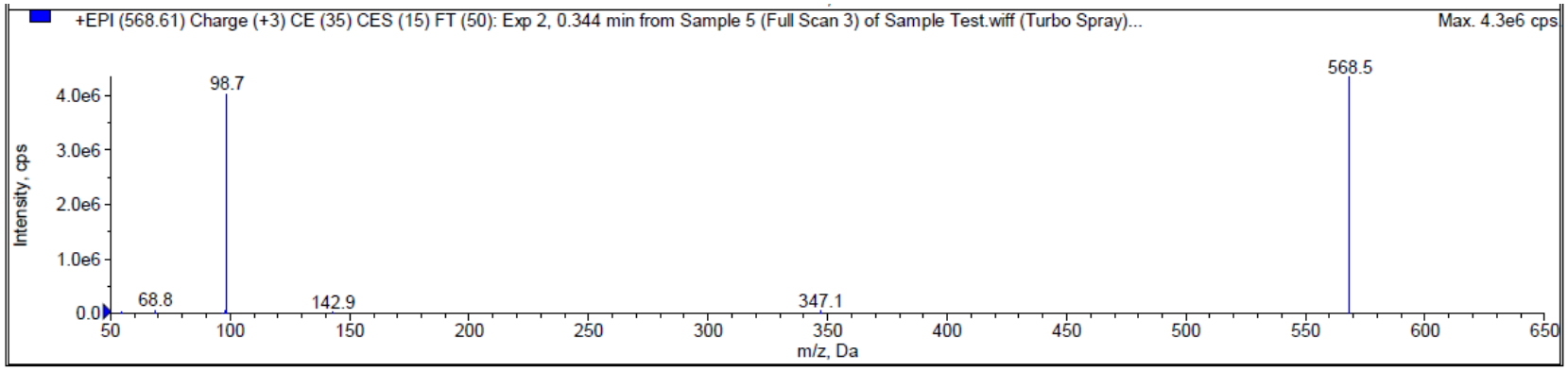
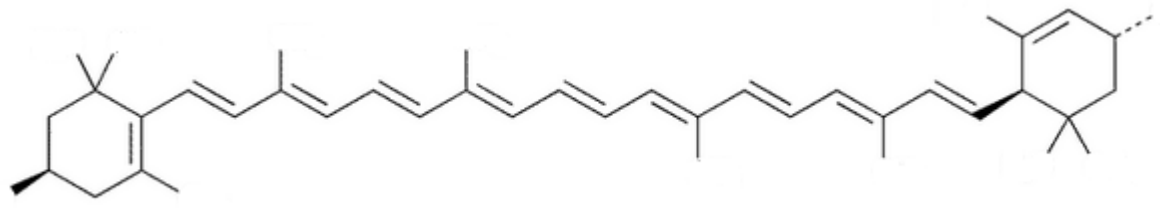


Fig 5.6 Mass spectrum of lutein in the yellow extract from *Ipomoea batatas* leaves

Structure of the lutein molecule:

$C_{40}H_{56}O_2$



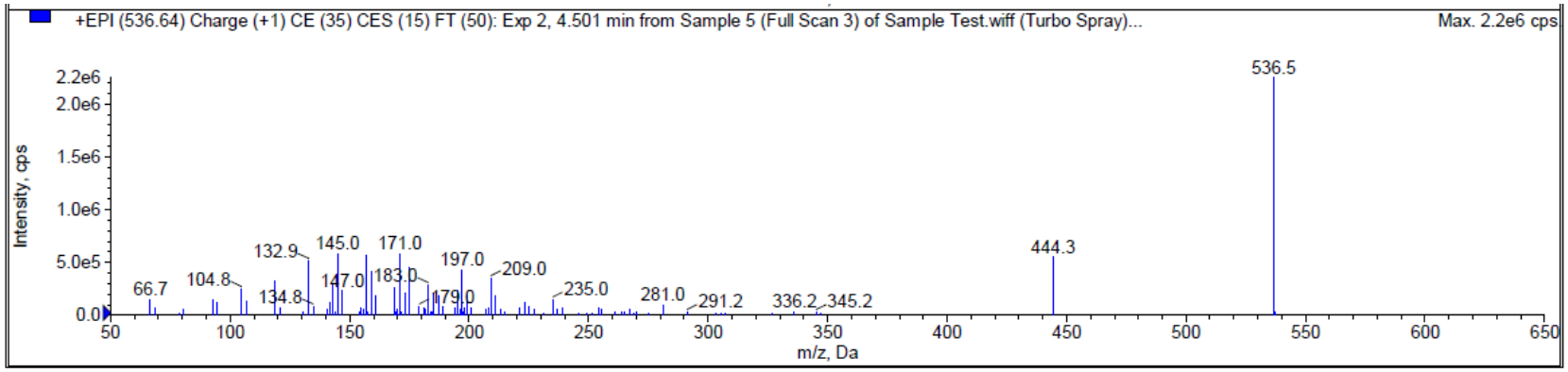
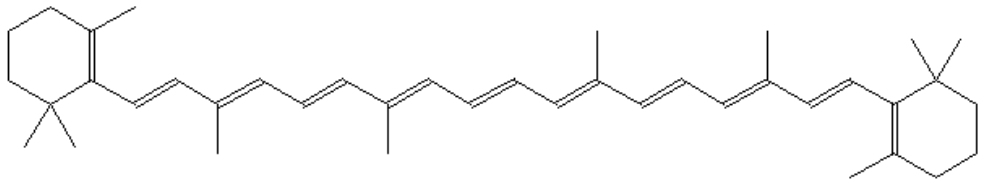


Fig 5.7 Mass spectrum of β-carotene in the yellow extract from *Ipomoea batatas* leaves

Structure of the β-carotene molecule:

$C_{40}H_{56}$



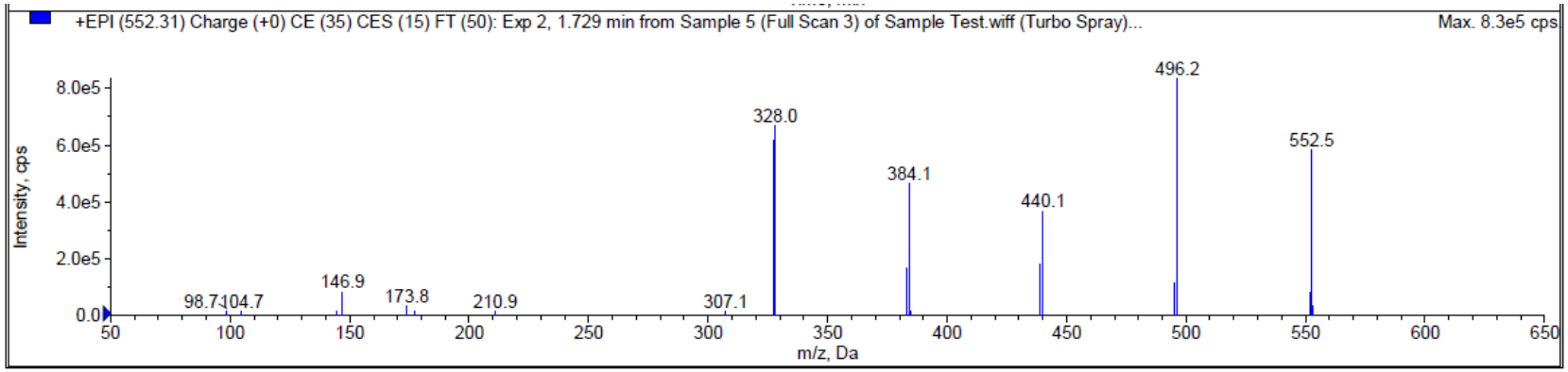
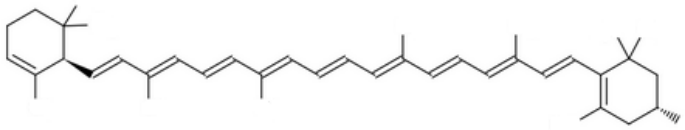


Fig 5.8 Mass spectrum of beta cryptoxanthin in the yellow extract from *Ipomoea batatas* leaves

Structure of the beta cryptoxanthin molecule:

$C_{40}H_{56}O$



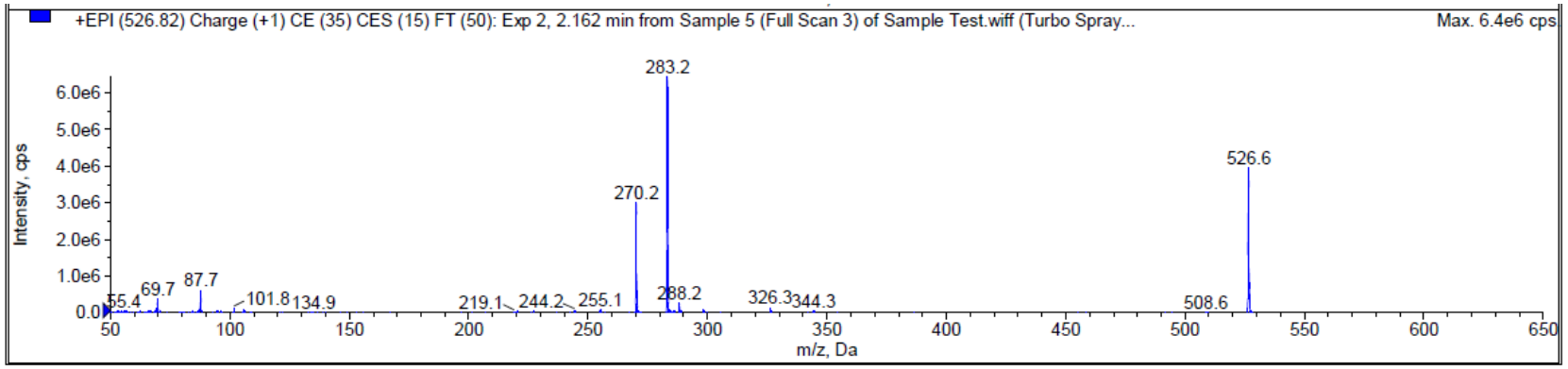
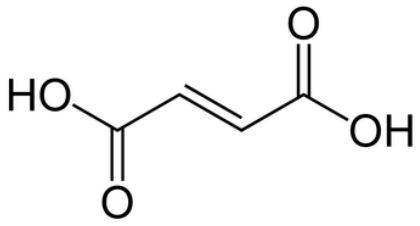


Fig 5.9 Mass spectrum of fumaric acid in the yellow extract from *Ipomoea batatas* leaves

Structure of the fumaric acid molecule:

$C_4H_4O_4$



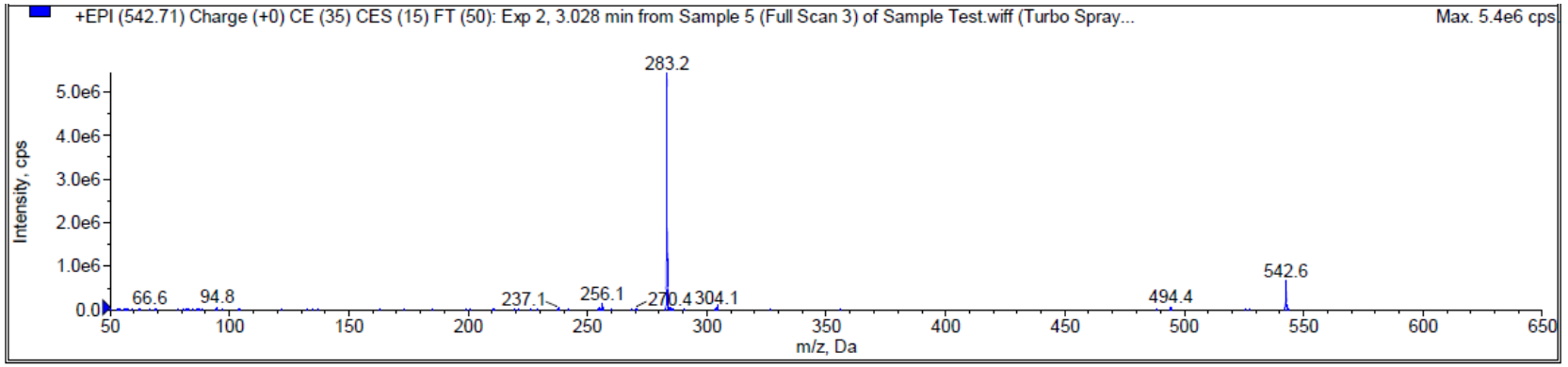
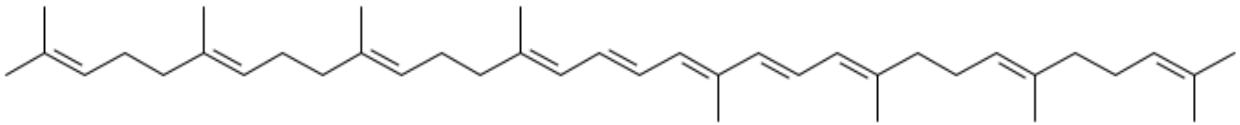


Fig 5.10 Mass spectrum of phytofluene in the yellow extract from *Ipomoea batatas* leaves

Structure of the phytofluene molecule:

$C_{40}H_{62}$



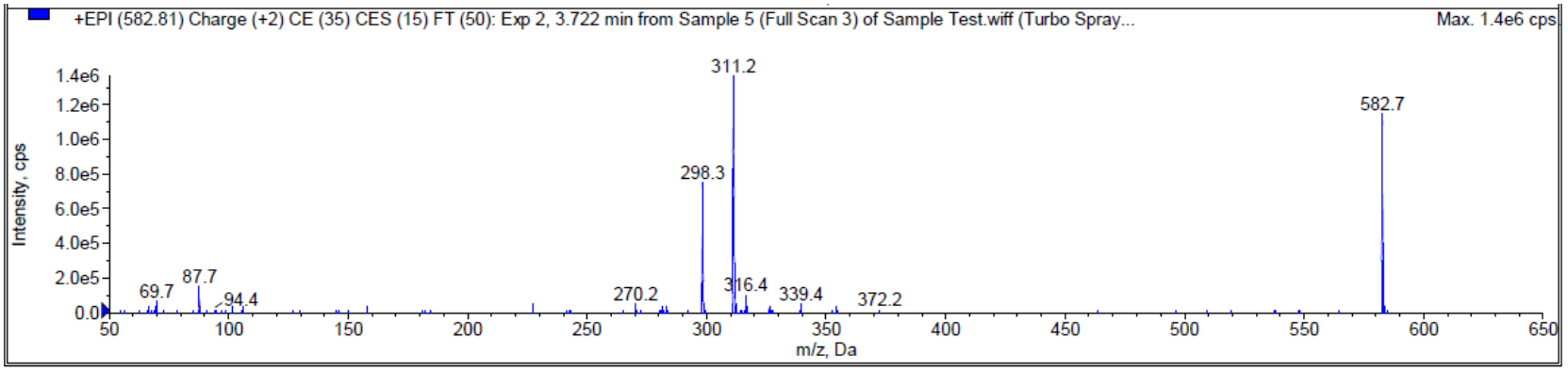
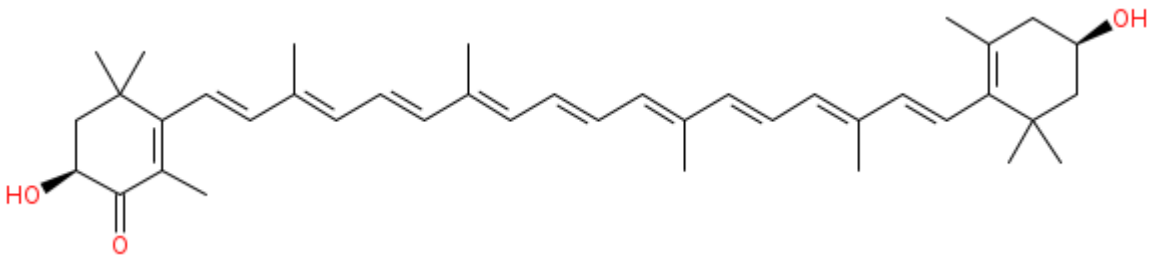


Fig 5.11 Mass spectrum of 4-ketozeaxanthin in the yellow extract from *Ipomoea batatas* leaves

Structure of the 4-ketozeaxanthin molecule:

$C_{40}H_{54}O_3$



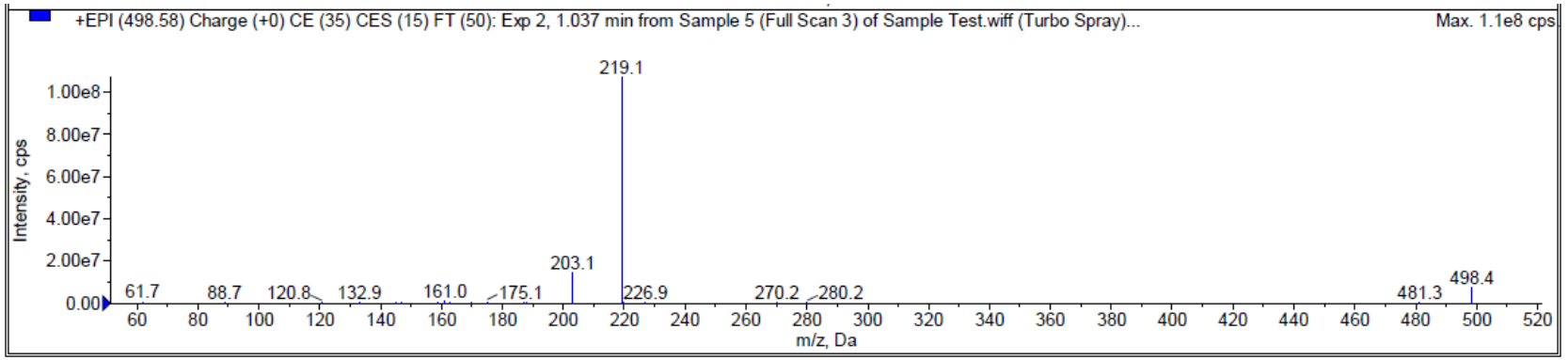
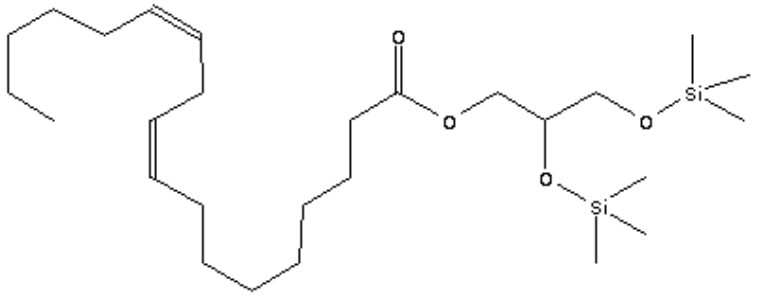


Fig 5.12 Mass Spectrum of 1-monolinoleoylglycerol trimethylsilyl ether in the yellow extract from *Ipomoea batatas* leaves

Structure of the 1-monolinoleoylglycerol trimethylsilyl ether molecule:

$C_{27}H_{54}O_4Si_2$



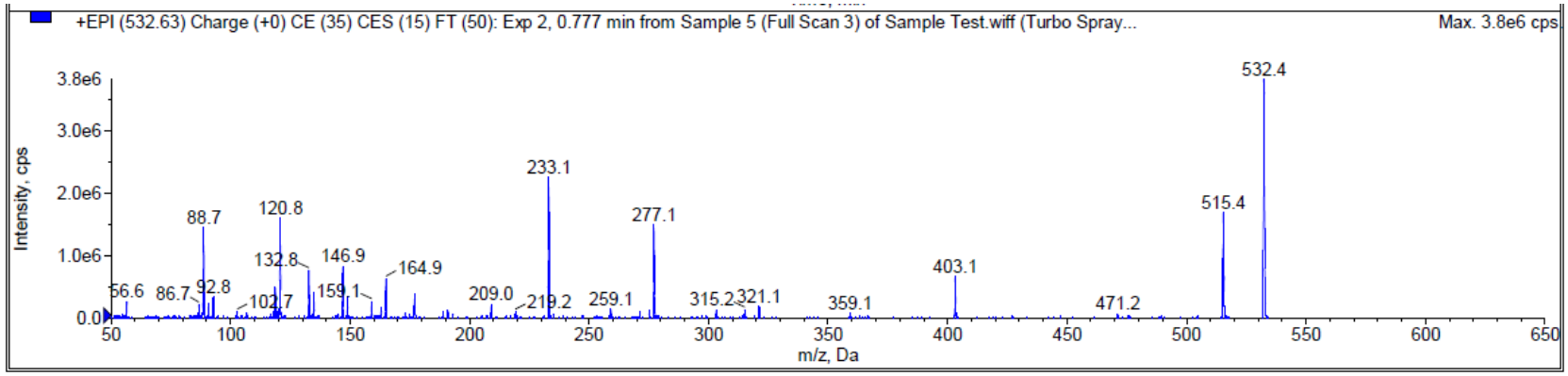
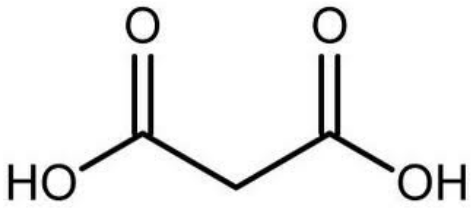


Fig 5.13 Mass Spectrum of malonic acid in the yellow extract from *Ipomoea batatas* leaves

Structure of the malonic acid molecule:

$C_3H_4O_4$



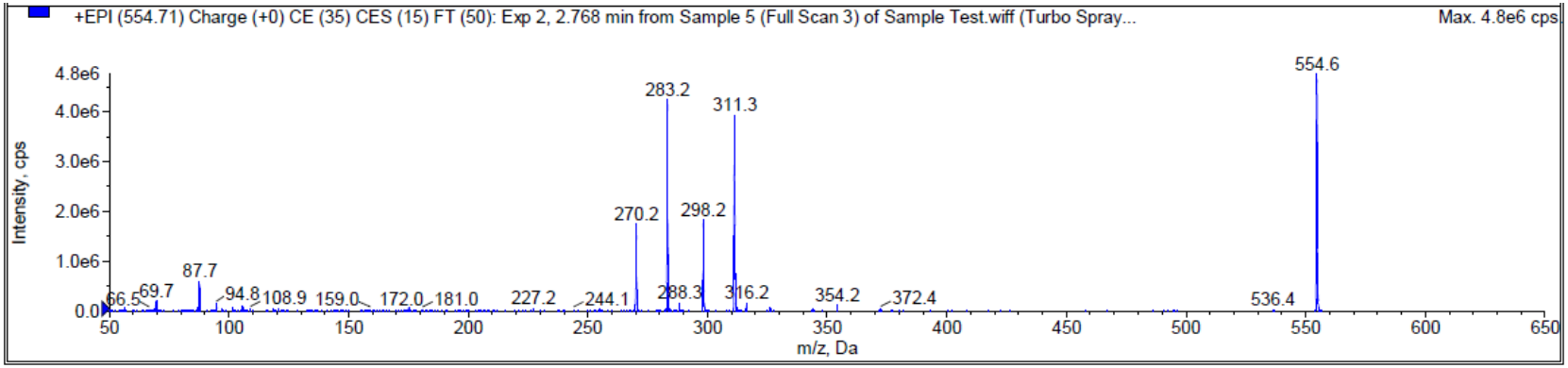
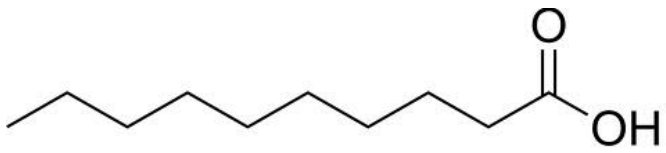


Fig 5.14 Mass Spectrum of decanoic acid in the yellow extract from *Ipomoea batatas* leaves

Structure of the decanoic acid molecule:

$C_{10}H_{20}O_2$



5.3.2 Pesticide Analysis on Yellow Pigments Extract

Table 5.6 shows the results of the pesticides analyses for the detection of organophosphorus insecticide, organochlorine insecticide and herbicide residues in the yellow extract. No pesticides were detected for the three types of screenings (detection limit 1 ppm) in the yellow extract.

Table 5.6 Pesticide Analyses on Yellow Pigments Extract

Types of Pesticide Screening	No. of Pesticide Screened	Results (Detection limit 1 ppm)
Organophosphorus Insecticide	136	No detection
Organochlorine Insecticide	52	No detection
Herbicide	251	No detection

5.3.3 Total Polyphenols (Folin-Ciocalteau) Assay

Total polyphenols in the yellow extract were determined in terms of gallic acid equivalent (GAE). Based on the standard curve (Figure 5.15), total polyphenols content in the yellow (carotenoids) extract and leaf extract was calculated as 2.99 ± 0.08 g/100g GAE and 3.47 ± 0.02 g/100g GAE respectively (n=3).

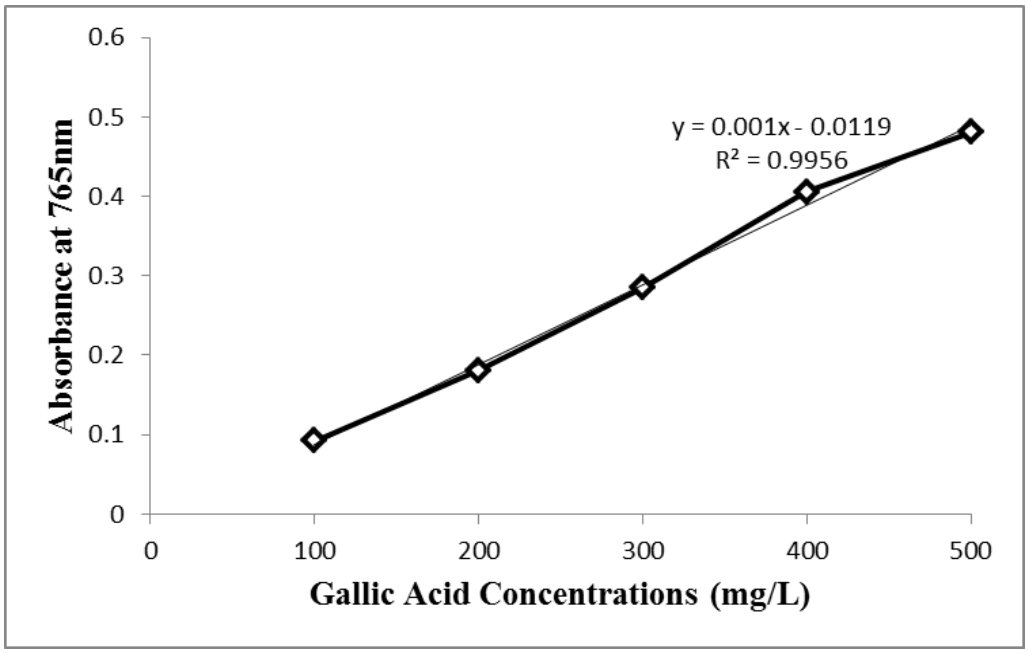


Fig 5.15 Standard curve for gallic acid used in the Folin-ciocalteau assay

5.3.4 Vanillin-HCl Assay

Total flavonoids content in the extracts was calculated based on the catechin standard curve (Figure 5.16). The concentration of total flavonoids in the leaf extract was 144.60 ± 40.50 µg/g catechin equivalent whereas the content in the yellow extract was slightly lower at 114.86 ± 4.35 µg/g catechin equivalent (n=3).

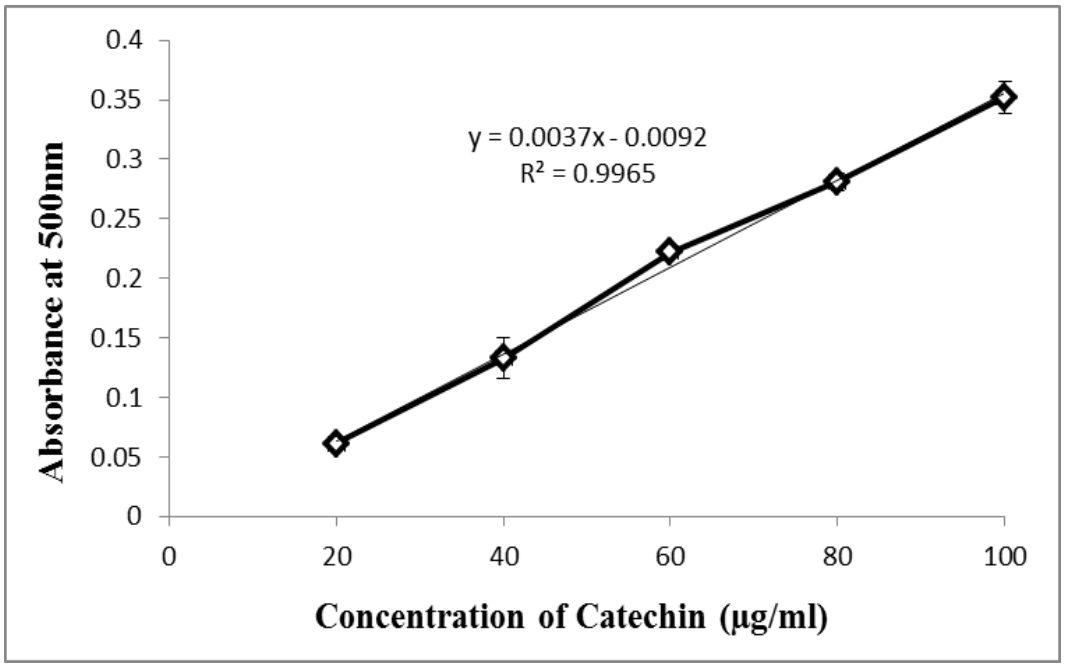


Fig 5.16 Standard curve for catechin used in vanillin-HCl assay

5.3.5 DPPH Radical Scavenging Assay

The radical scavenging activity of the different concentrations of vitamin C, leaf extract and yellow extract from *Ipomoea batatas* var. Oren leaves is shown in Figure 5.17 (n=3). The yellow extract contained slightly lower amount of radical scavenging activity compared to vitamin C except for at 600µg/ml. Vitamin C standard showed the highest scavenging activity (IC_{50}= 471.6 ± 0.4 µg/ml), followed by carotenoids extract (IC_{50}= 491.86 ± 0.3 µg/ml) and leaf extract (IC_{50}= 545.39 ± 0.4 µg/ml). The percentage of inhibition in this case is inversely proportional to the radical scavenging activity of the extract; vitamin C has the highest scavenging activity compared to the other extracts.

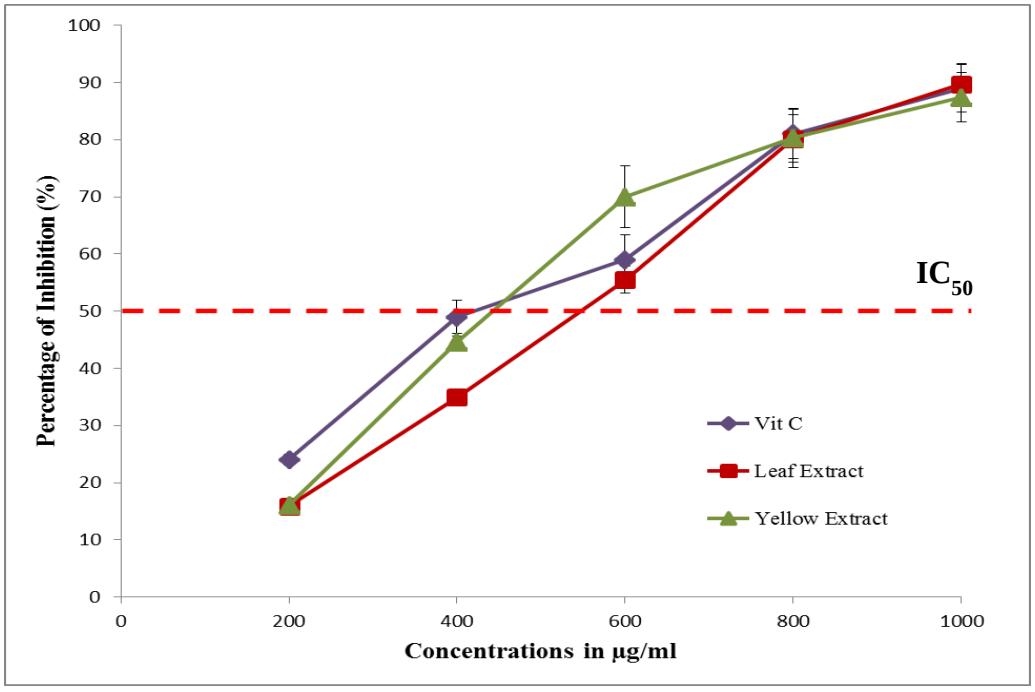


Fig 5.17 Radical scavenging activity of the vitamin C, leaf extract and yellow extract from *Ipomoea batatas* leaves depicted by the percentage of inhibition

5.3.6 Reducing Power Assay

The reducing power of the extracts at the different concentrations is shown in Figure 5.18. It was observed that the reducing powers for both the extracts were lower compared to the BHA standard. The leaf extract has slightly higher reducing power compared to the yellow extract.

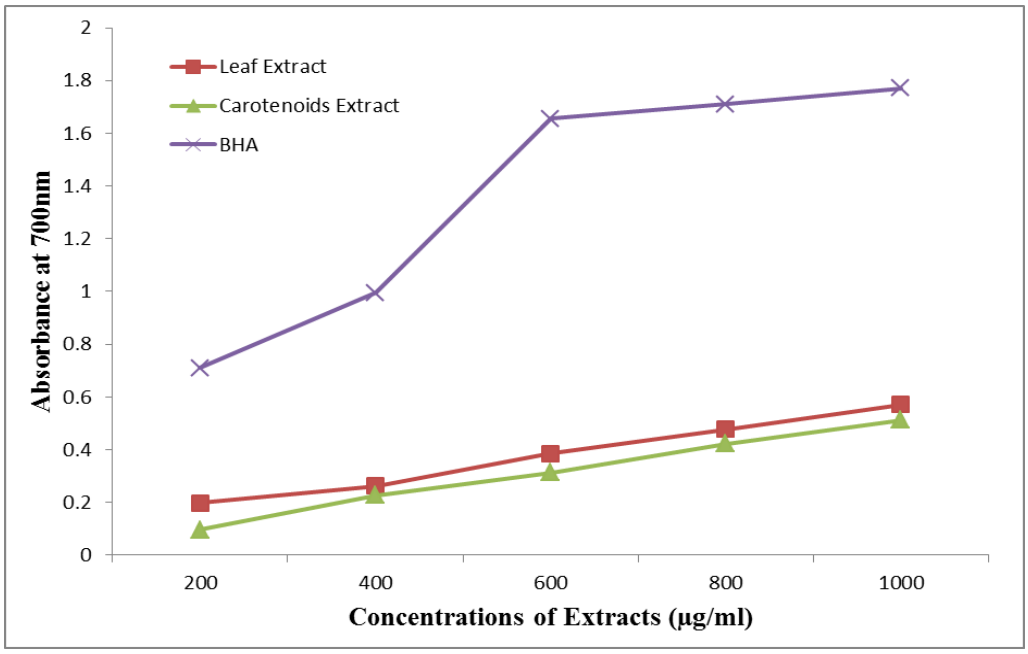


Fig 5.18 Reducing power of the leaf and yellow extract from *Ipomoea batatas* leaves

5.4 DISCUSSION

The yellow (carotenoids) extract from the *I. batatas* var. Oren leaves was analysed using the liquid chromatography mass spectrometry/ mass spectrometry (LCMS/MS) method. The concentrations of the β-carotene and lutein pigments in the yellow extract were determined using the standards. β-carotene concentration in the yellow extract was found to be 10.8 mg/100g FW while result obtained from the spectrophotometric method showed that the concentration of β-carotene pigments in the extracted sample was 10.738 mg/100g FW.

On the other hand, the concentration of the lutein pigments was 10.0 mg/100g and 9.8 mg/100g FW using the LCMS/MS and spectrophotometric methods respectively. Only a small difference was observed in the pigment concentration for both the β-carotene (0.1 mg/100g FW) and lutein (0.2 mg/100g FW) pigments when the two methods were used. This concluded that the spectrophotometric method used was a reliable, accurate and less time-consuming method to determine the concentrations of the pigments in the yellow extract. While lutein contents in *I. batatas* leaves have been studied in the leaf extract, no studies have been conducted to measure the lutein and β-carotene pigments in the carotenoids extract from leaves (without chlorophyll).

Previous study conducted on kale leaf showed that cultivar types and seasonal variations contributed significantly to the differences in the carotenoids (lutein, β-carotene, vitamin A and total carotenoids) contents, with leaves harvested during the winter season showed higher carotenoids content compared to leaves harvested during the summer season (Young and Britton, 1990; Mercadante and Rodriguez-Amaya, 1991). Previous studies also found that lutein content in leaves extract from different cultivars and growth locations varied from 10 to 58 mg/100g FW respectively

(Menelaou, 2006; Ishiguro and Yoshimoto, 2007). The lower β-carotene and lutein level found in the *I. batatas* var Oren leaves observed may followed the similar pigments pattern as in kale leaves (lower pigments content in tropical area due to high temperature). However, this interesting pattern seems only highly applicable in green leaves as the β-carotene level in fruits was found to be higher during the summer season. Hence, sunlight might not have effects on the carotenoids in fruits, which consist of non-photosynthetic tissues (McCollum, 1954; Watada *et al*., 1976).

Rapid screening for bioactive carotenoids was performed to determine the compounds present in the yellow extract based on their molecular weights and characteristic fragmentation patterns. In the ESI ionisation process, traces of formic acid are usually added in the positive ionisation mode to aid protonation of the sample molecules. For the negative ionisation mode, traces of ammonia solution or a volatile amine is often added to aid in the deprotonation of the sample molecules. β-carotene pigment was detected at 536 amu when analysed using LCMS/MS, whilst lutein was indicated by the 568 amu fragment, which showed consistency with the previous reported m/z value for lutein (Guaratini *et al*., 2005). The variation in the m/z value of a compound is usually due to the addition or loss of a proton. Besides β-carotene and lutein pigments, analyses using the LCMS/MS method also detected the presence of other compounds, which was a mixture of different types of carotenoids, organic acids, waxes, and oils that were naturally occurring in plant materials. The m/z value of the compound obtained from the mass spectrum was used to search against the online database (NIST Chemistry Webbook) to identify the particular compound.

Besides the main carotenoid pigments such as β-carotene and lutein, beta cryptoxanthin and 4-ketozeaxanthin were also detected in low concentrations in the extract. 4-ketozeaxanthin an isomer of the lutein pigment that has undergone degradation or isomerization which was probably contributed by several factors such as exposure to light, oxygen, high temperature and harsh treatment during extraction and processing. Phytofluene on the other hand is a carotenoid precursor involved in the initial step of the carotenoids biosynthesis pathways. The colourless feature of phytofluene is contributed by the lower number of double bonds present in the C40 carbon chain and its presence is often related to the co-existence of other carotenoids in plant tissues (Zechmeister and Sandoval, 1946). Studies have shown that phytofluene often work in synergy with lycopene and helps in the protection of plant cell from damages (Engelmann *et al*., 2011). However, lycopene was not detected in the yellow extract.

Common plant organic acids such as malonic acid, decanoic acid, fumaric acid and benzenemetricarboxylic acid were also detected in the yellow extract by the LCMS/MS analysis. This group of organic acids are intermediates in the tricarboxylic acid cycle involved in the fatty acids production in plants. The accumulation of organic acids in plant tissue is mostly related to their vital functions in the transportation of micronutrients, the production of photosynthetic intermediates and the adaptation to stress by the plant (Anderson *et al*., 1993; Jones, 1998; Lopez-Bucio *et al*., 2000). On the other hand, plant oils such as oleoyl and linoleoyl were also detected and might possibly play an important role in maintaining the stability of pigments in the extracted sample. However, no detection was observed for the negative ionisation mode.

The natural dye industry has been viewed as an upcoming and lucrative industry given the right source and technology for commercial dye production. According to the specification laid by the EU legislation Directive 2008/128/EC for lutein (E 161B), a minimum of 4% lutein must be present from the total content of the natural colourant to be eligible as a source of natural colourant. Another natural yellow colourant, named 'mixed carotenes' (E160a) has also been authorised by the EU with the main colouring consisting of carotenoids (β-carotene instead of lutein). Under the current regulations, the authorised mixed carotenoids with lutein as the major pigments are obtained from alfalfa via solvent extraction, which includes the saponification process for chlorophylls removal (JECTA, 2006).

Currently, the commercial extraction of lutein is from marigold (*Tagetes erecta* L.) flowers, which are grown widely in Africa. However, other alternative sources have been studied widely due to the low lutein content in the flowers (approximately 0.03% dry weight) (Sánchez *et al*., 2008). For instance, microalgae have been proposed as the suitable source for lutein extraction in some studies. Several strains of microalgae such as *Muriellopsis* sp. (Del Campo *et al*. 2001, 2007), *Chlorella zofingensis* (Del Campo *et al*. 2000) and *Scenedesmus almeriensis* (Sánchez *et al*. 2007) have been studied and used for extraction due to their high content of lutein. However, these sources required the opening of lands, costly labour and special equipment for cultivation and thus make natural dye production less economical and desirable to be used for industrial applications.

Previous studies by Khachatryan *et al*. (2003) had suggested the usage of *I. batatas* leaves as a potential source of lutein for food fortification as a means for preventing age-related macular degeneration (AMD). In his study, it can be concluded that lutein levels in the *I. batatas* leaves ranked the highest of all the edible green vegetables and only second to the African Marigold (*Tagetes erecta*). However, the analyses were performed via chromatography (HPLC) on the leaves extract of the *I. batatas* and the proper extraction method for the carotenoids from the leaves was not established. Besides lutein, *I. batatas* leaves have also been reported to contain high amount of nutrients and polyphenol, which are important in the human diet (Ishiguro and Yoshimoto, 2007).

The main concern of using leaf as source of extraction is the remaining pesticides residues in the final product after processing. The ultimate goal especially in the food industry is a pesticide free final product after the extraction and manufacturing process. Pesticides are used in the *I. batatas* farming to ensure the healthy growth of leaves and storage roots. In this study, gas chromatography coupled with mass spectrometry method (GCMS) was used to screen for the presence of pesticides in the yellow pigments extract. Analyses using GCMS revealed that herbicides, organophosphorus insecticides and organochlorinated insectides were not detected in the sample extract, which may possibly contributed by the liquid-liquid partitioning process in removing pesticide residues from the yellow extract. This showed that the extraction method used in this study is good in removing pesticides residues from the final extract.

Some of the naturally found antioxidant in plants includes vitamins, phenolics, flavonoids, anthocyanin, dietary glutathione and endogenous metabolites (Larson, 1988; Cao *et al*., 1997). The antioxidant activity in the yellow extract from the *Ipomoea batatas* var. Oren leaves was determined using several colorimetric assays. Fresh leaves

were used because previous studies have shown higher amount of antioxidants in fresh samples compared to oven dried and air-dried samples (Jia *et al*., 1999). Besides, the effect of the extraction method, the antioxidant properties of yellow extract was determined and compared with that of the leaf extract.

The phenolics are well-known group of secondary metabolites that comprise of a large group of biologically active compounds (Dreosti, 2000). The general sources of polyphenols are grains (peas, barley and other legumes), fruits (cranberries, strawberries), vegetables (cabbage, onion, parsley) as well as tea. Polyphenols help to maintain a healthy body by scavenging or removing the reactive oxygen species (ROS) and free radicals in the body. The study of phenolics compound in food has been intensive after research on red wine showed that moderate consumption of red wine could help in the prevention of cardiovascular diseases due to the presence of polyphenols (Marinova *et al*., 2005). The antioxidant properties of the phenolics compounds are due to their phenolic hydroxyl groups that have the ability to scavenge radicals (Sawa *et al*., 1999). Previous studies have reported that genetics factors, environment conditions, germination, degree of ripening and plant varieties are factors that can influence the phenolics level in plants (Bravo, 1998). Presence of polyphenols therefore strongly suggests the antioxidant potential in the yellow extract.

The Folin-Ciocalteau method was used to determine the total polyphenols contents in the yellow extract. Upon the detection of polyphenol, the Folin-Ciocalteau reagents changes from yellow to blue due to the chemical reduction of tungsten and molybdenum oxides mixture in the reagent. The changes in colour formation was determined at 765 nm and the total polyphenol content in the extract was calculated based on the gallic acid standard calibration curve ($y = 0.001x – 0.0119$, $R^2 = 0.9956$) in terms of gallic acid

equivalents (GAE). The total polyphenols content in the leaves extract was 3.47 ± 0.02 g/100g GAE whereas the yellow extract estimated a lower total polyphenols of 2.99 ± 0.08 g/100g GAE.

Previous studies conducted on olive pulp revealed that the highest concentration of total polyphenol was detected in the Mishen olive cultivar with approximately 2.99 ± 0.36 g/100g GAE (Hajihmahmoodi *et al*., 2008). In comparison, the yellow extract in this study showed almost a similar amount of total polyphenol content with the olive pulp extract. Previous study on *I. batatas* leaves showed that the total polyphenol content in the different cultivars ranged between 1.42 to 17.1 g/100g DW (Islam *et al*., 2002) while the leaf extract in this study showed a value of approximately 3.470 ± 0.024 g/100g GAE. Geographical area of cultivation has been found to influence the antioxidants composition level in *Cichorium intybus* leaves thereby providing an important insight into the differences in the total polyphenol levels found in the *I. batatas* var. Oren leaves (Schaffer *et al*., 2005).

In addition, one previous study on *Poacynum henersonii* had also shown variations in the total phenolic contents in samples collected from three different sites in China besides the seasonal variations (Ma *et al*., 2003). To sum up, the storage conditions, storage time, time of harvesting and transportation method may influence the level of phytochemicals composition in the plants. Aside from these factors, interference from a number of sources such as sugar and sulphur dioxide might have an impact on the assay. Time limitation for the reaction is also a factor due to the number of samples that can be handled at one time.

Flavonoid is one of the major groups besides isoprenoids and alkaloids that make up the secondary metabolites produced by higher plants. Flavonoids are well-known by their blue, red and purple pigments in fruits, flowers and leaves. Flavonoids content in fruits, vegetables and food consist of quercetin, kaempferol, apigenin, luteolin and myricetin (Hertog *et al*., 1993). The flavonoids are subdivided into several families such as the flavonols, flavones, isoflavones, anthocyanidins and others.

Catechin is a flavonoid, which contains two benzene rings in its structure and was found to be the most powerful scavenger and therefore, often used as standard to measure the content of flavonoids. The structure of flavonoids has been known to contribute to the antioxidant properties of these compounds. Green leafy vegetables are known to contain high amount of antioxidants activity which are partially contributed by the presence of flavonoids. Besides, tannins can be beneficial to humans due to their antibacterial and therapeutic nature (Watson and Preedy, 2008).

Flavonoid contents were documented at 144.6 ± 40.5 µg/g (or 144.6 ± 40.5 mg/kg) catechin equivalent and 114.86 ± 4.35 µg/g (114.86 ± 4.35 mg/kg) catechin equivalent for the leaf extract and yellow extract respectively. Statistical analyses concluded that the difference between the two extracts was not significant and therefore the extraction method used for the yellow extract was able to retain the flavonoids contents from the leaves. A study conducted by Koo and Mohamed (2001) concluded that highest total flavonoids content was found in black tea (1491.0 mg/kg) and papaya shoots (1264.0 mg/kg) among 62 types of vegetables extracts studied. On the other hand, commonly consumed vegetables such as soybean sprout (78.5 mg/kg), red spinach (29.5 mg/kg) and kailan (14.5 mg/kg) showed lower flavonoid content compared to the yellow extract from the *Ipomoea batatas* leaves. This showed that the yellow extract from the *I. batatas* var. Oren leaves can be utilised as a good source of yellow colouring with the

presence of flavonoids. A major precaution in this assay is that the catechin standards used must be kept in tightly sealed bottle and stored in refrigerator to maintain the accuracy of the analysis.

It was observed that the yellow extract contained slightly higher scavenging activity compared to the leaf extract at all concentrations. At the concentration 600 µg/ml, scavenging activity of the yellow extract (approximately 70% inhibition) was observed to be higher than vitamin C (approximately 60% inhibition), a strong radical scavenger. Overall, the yellow extract showed almost similar radical scavenging activity compared to the vitamin C standard in this study. The higher radical scavenging activity of the yellow extract compared to the leaf extract showed that the extraction process did not caused pigments degradation in the extract.

However, several factors were found to affect the scavenging activity of the sample extract namely the polarity of the reaction medium, the chemicals structure of the radical scavenger (the antioxidants) and pH of the reaction mixture (Saito *et al.*, 2004). Besides that, DPPH method used is also very sensitive to light and pH and hence precautionary steps need to be taken when conducting this analysis (Sharma and Bhat, 2009).

The reducing ability of the leaf extract and yellow extract from the *I. batatas* leaves at the different concentrations were compared with butylated hydroxyl anisole (BHA), a synthetic antioxidant. The BHA showed overall higher reducing power followed by the leaf extract and yellow extract. The ability to scavenge free radicals is an additional feature of the yellow extract, which can help to maintain and preserve the good condition of food in food industry.

In conclusion, the composition of the yellow extract was successfully determined via the LCMS/MS analysis. Besides, the presence of other compounds such as organic acids, lipids and carotenoids in the yellow extract were also determined from their mass spectra. The extraction method used was found to be effective in removing pesticides residues from the yellow extract. Comparison between the leaf and yellow extract from *Ipomoea batatas* var. Oren leaves showed that the latter had retained most of its antioxidants properties after the extraction process. The antioxidant properties, total polyphenols and flavonoids contents will be a value added benefit for the yellow extract. With the success in producing this extract, we hope that the cost of production for natural yellow dye can be lowered in the market so that more consumer can engaged in the usage of natural dye for better health and lifestyle.

CHAPTER 6

Isolation and Characterisation of Lycopene Epsilon-Cyclase (*LcyE*), Lycopene Beta-Cyclase (*LcyB*) and Phytoene Synthase (*Psy*) Genes from *Ipomoea batatas* Leaves

6.1 INTRODUCTION

Carotenoids are pigments produced within the plastids from the carotenoid biosynthesis pathway and involve many genes. Identification of these genes may provide clearer insight on the carotenoid biosynthesis in plants and the study on the molecular pathways started with the successful isolation of enzymatic genes involved in the pathway. Previous studies on these enzymes (such as lycopene β-cyclase and lycopene ε-cyclase) have been limited because they are not abundant in plants and are very labile during extraction and purification (Hirschberg, 2001).

Gene studies are important to identify the enzymes involved in the production of carotenoids. Phytoene synthase (*Psy*) is a membrane bound enzyme which catalyses the condensation of two geranylgeranyl pyrophosphate to produce the 15-cis phytoene. *Psy* was found to be the rate-limiting enzyme in the carotenoid biosynthesis pathway in ripening fruits (tomato), seeds (canola) and flowers (marigold) (Bramley *et al*., 1992; Fraser *et al*., 1994; Shewmaker *et al*., 1999; Moehs *et al*., 2001). This feature therefore enables the usage of PSY as a key regulator in the carotenoid biosynthesis.

Another important step in the biosynthesis cycle is the cyclisation of lycopene, which branches out to form carotenes and its derivatives xanthophylls. The lycopene β-cyclase (*LcyB*) enzyme catalyses the two-way reactions for one β-ionone ring at each end of the lycopene to produce the β-carotene molecule (Cunningham and Gantt, 2001). On the other hand, lutein, the predominant carotenoid in photosynthetic tissues has a second type of cyclic end group known as the ε-ring (catalysed by the lycopene ε-cyclase (*LcyE*) enzyme) and results in lutein containing an ε-ring at one end and β-ring at the other end (Goodwin, 1980). The genes of interest in this study are shown in Figure 6.1 and marked in red.

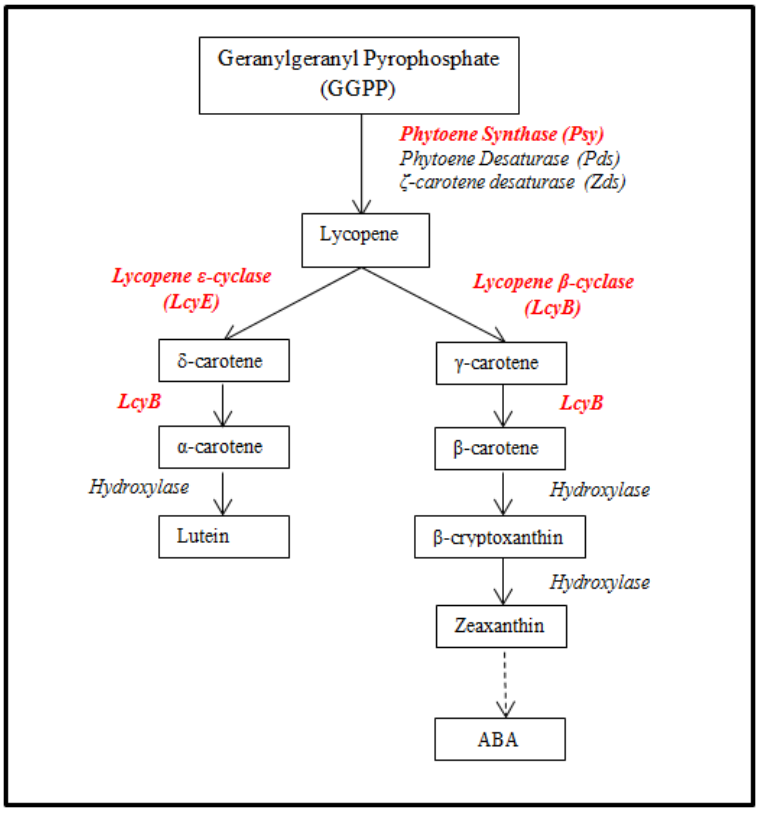


Fig 6.1 Simplified carotenoid biosynthesis pathway in plants. The genes of interest are marked in red.

Hence, the objectives of this chapter are to extract the total RNA from *Ipomoea batatas* leaves as a starting material for molecular study and to isolate the lycopene ε-cyclase (*LcyE*), lycopene β-cyclase (*LcyB*) and phytoene synthase (*Psy*) genes involved in the carotenoids biosynthesis cycle using Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) method. The isolated genes would be characterised using bioinformatics tools.

6.2 MATERIALS AND METHODS

6.2.1 Sample Preparation

Young leaves as shown in Figure 6.2 (approximately 2 to 3 days old) were harvested, washed under running water to remove dirt and air dried at room temperature. The leaves were weighted and ground immediately using mortar and pestle under liquid nitrogen prior to RNA extraction.

Fig 6.2 Young leaves of *I. batatas* var. Oren used in RNA extraction

6.2.2 RNA extraction from *Ipomoea batatas* leaves

Total RNA was extracted using Qiagen RNeasy Plant Mini Kit according to the manufacturer's protocols as described below. Approximately 100 mg of ground leaves powder was transferred into a 1.5 ml microcentrifuge tube containing 450 µl of RLT Buffer (Qiagen) and 4.5 µl of β-mercaptoethanol (99 %) (Sigma). The mixture was vortexed vigorously and transferred to a QIA Shredder Spin Column placed in 2 ml collection tube and was centrifuged at 12000 rpm for 2 min at room temperature using a microcentrifuge (UEC). The flow through was transferred to a new microcentrifuge tube

and 200 µl of ethanol was added and mixed by pipetting. The sample (including the precipitate) was transferred to RNeasy Spin Column placed in a 2 ml collection tube and centrifuged at 10000 rpm for 15 sec at room temperature. The flow through was discarded and the collection tube was re-used.

RW1 buffer (700 µl) was added to the RNeasy Spin Column and was centrifuged at 10000 rpm for 15 sec at room temperature to wash the spin column membrane. The flow through was then discarded and 500 µl of Buffer RPE was added into the column. The column was centrifuged at 10000 rpm for 15 sec and the flow through was discarded. RPE Buffer (500 µl) was added again but this time the column was centrifuged for 2 min at 10000 rpm. The RNeasy spin column was placed in a new 2 ml collection tube and was centrifuged at 12000 rpm for 1 min to remove traces of RPE buffer. The spin column was then placed in a new 1.5 ml collection tube and 40 µl of RNase-Free water was added to the centre of the membrane and was incubated for 5 min before centrifugation at 10000 rpm for 1 min to elute the RNA. This step was repeated twice. Two microliters of DNase (New England) was added to the RNA and was incubated for 10 min at room tempersture to remove any trace DNA in the sample. The RNA was stored at -20ºC until further use.

6.2.3 Quantitative Analysis of RNA

The RNA was quantified using a UV-Vis spectrophotometer (Bio-ray) and absorbance was measured at 260 nm and 280 nm. The purity of the RNA was determined using the A_{260}/A_{280} ratio method. The concentration of the extracted RNA was calculated based on the following formula (Qiagen, 2011):

$$\text{Concentration RNA sample (µg/ml)} = 40 \times A_{260} \times \text{dilution factor}$$

6.2.4 Primer Design for RT-PCR

The *LcyE*, *LcyB* and Psy nucleotide gene sequences from different plants were retrieved from the GenBank (www.ncbi.nlm.nih.gov/genbank/). The conserved sequence among the different plants was used to design the specific primer for amplification process. Oligo Analyzer 1.5 (GeneLink ^{TM}) software was used to assist in the primer design. Table 6.1 shows the primers' names, sequences and the source used in designing the primers.

Table 6.1 Primers Used in RT-PCR

Gene	Primer Name	Primer Sequence	Source	Genebank Accession Number
Lycopene epsilon cyclase (*LcyE*)	LcyEF	5'TGC ATT GAA CAT GTT TGG 3'	*Ipomoea sp. Kenyan* & *Solanum lycopersicum*	AB499054.1 & EU533951.1
	LcyER	5' GAA KGC AAA TAA CAT GAG GTC 3'		
Lycopene beta cyclase (*LcyB*)	LcyBF	5' GCW GTT AAA GCT AGT RCC T 3'	*Ipomoea sp. Kenyan & Lycopersicon esculentum*	AB499055.1 & X86452.1
	LcyBR	5' CCT GTA ACA AAT TGT TGA TC 3'		
Phytoene Synthase (*Psy*)	PsyF	5' GGG CGT GTT GAG TGA AGC 3'	*Ipomoea sp. Kenyan & Solanum lycopersicum & Lycopersicon esculentum*	AB499050.1 & EU734550.1 & L23424.1
	PsyR	5' GAG GTG AAG TAG TTG TTG ATG 3'	*Ipomoea sp. Kenyan*	AB499050.1

6.2.5 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was carried out using the AccessQuick ™ RT-PCR Master Mix 2X (Promega) using the following combination:

Table 6.2 Reagents Used in RT-PCR

Reagents	Volume (µl)	Concentration
AccessQuick ™ RT-PCR Master Mix 2X	25	1X
Forward primer	3	0.6 µm
Reverse primer	3	0.6 µm
RNA Template	10	1 µg/µl
Nuclease free water	9	-
Total volume	50	

The reaction mixture was added with 1 µl (5U) of AMV Reverse Transcriptase. The RT-PCR cycle was performed using a thermocycler (Biorad) and the conditions used for amplification are shown below:

Reverse transcription: 45ºC (45 min)

Denaturation: 95ºC (2 min)

95ºC (1 min)

Annealing temperature (2 min) } 40 cycles

72ºC (1 min)

Extension: 72ºC (5 min)

Hold: 16ºC (∞)

RT-PCR product was confirmed through agarose gel electrophoresis. The 1% agarose gel was prepared by adding 0.5 g of agarose powder (Promega) to 50 ml of 1% TAE buffer (Promega). The mixture was brought to boil and was cooled to approximately 50ºC before the addition of 3 µl of ethidium bromide. The mixture was poured into a gel cast and left to solidify. Then, 4 µl of the PCR product was mixed with 1 µl of 6X Orange Blue loading dye (Promega) and loaded into the wells of 1% agarose gel. The gel was electrophoresed at 80V for 1.5 hours. The gel was viewed under UV trans-illuminator to observe the PCR products.

6.2.6 Purification of RT-PCR Product and Sequencing

Positive RT-PCR products were electrophoresed in 1% agarose gel for 1.5 hours at 80V. The specific band on the gel was excised and purified using Wizard® SV Gel & PCR Clean-Up System (Promega). An equal amount of membrane binding solution was added to the excised gel (100 µl: 0.1 g). The mixture was heated for 10 min in a 65 ºC water bath to solubilise the gel slice and later transferred to a SV Minicolumn. The column was centrifuged for 5 min at 10000 rpm at RT to bind the cDNA to the column membrane followed by the addition of membrane wash solution (700 µl) and centrifugation at 10000 rpm for 5 min at RT to remove impurities.

The flow through was discarded and another 500 µl of the membrane wash solution was added to the column and centrifuged at 10000 rpm for 5 min at RT. The column was placed in a 1.5 ml microcentrifuge tube and 40 µl of nuclease-free water was added to the centre of the membrane and left for 5 min before centrifugation took place at 10 000 rpm and RT for 5 min. The purified product was confirmed on a 1% agarose gel electrophoresed at 80V for 1.5 hours. The purified product was commercially sequenced by First Base Laboratories Sdn Bhd.

6.2.7 Sequence Analyses using Bioinformatics Tools

The RT-PCR product was analysed and characterised using bioinformatics tools from the following databases:

6.2.7.1 NCBI

BLASTp or Basic Local Alignment Search Tool Protein (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine the sequence homology between the isolated gene sequence with other sequences available in the database (Altschul *et al*., 1990).

6.2.7.2 ExPASy

The cDNA sequence of the gene obtained after RT-PCR was translated to amino acid sequence using the conversion tools provided by ExPASy (http://web.expasy.org/translate/) (Gasteiger *et al*., 2003). ScanProsite was used to detect the signature patterns or profiles that are present in the amino acid sequence using online tool (http://prosite.expasy.org/scan prosite/) (de Castro *et al*., 2006). On the other hand, amino acid sequences were analysed using ProtParam (http://web.expasy.org/protparam/) to determine its physico-chemical properties (Gasteiger *et al*., 2003). The structure of the amino acid sequence was then predicted using Swiss-Model software (http: //swissmodel. expasy.org/) and viewed using Rasmol software (Arnold *et al*., 2006).

6.2.7.3 Pfam

The conserved family domain of the protein sequence was determined from the available database in Pfam (http://pfam.sanger.ac.uk/) (Bateman *et al*., 2002).

6.2.7.4 ClustalW

The relationship between the translated amino acid sequences of *LcyE*, *LcyB* and *Psy* with sequences from other plants in the database were determined using ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and was summarised in a phylogenetic tree (Larkin *et al*., 2007).

6.3 RESULTS

6.3.1 Quantitative Analysis of RNA

The purity of the extracted RNA using the A_{260}/A_{280} ratio was recorded at 1.85, which falls under the pure category. Relative quantification revealed that the concentration of the RNA was approximately 37 µg/ml.

6.3.2 *LcyE* gene

6.3.2.1 Agarose Gel Electrophoresis

RT-PCR using the *LcyE* primers yielded a single product with a size between 600 bp to 650 bp as shown in Figure 6.3.

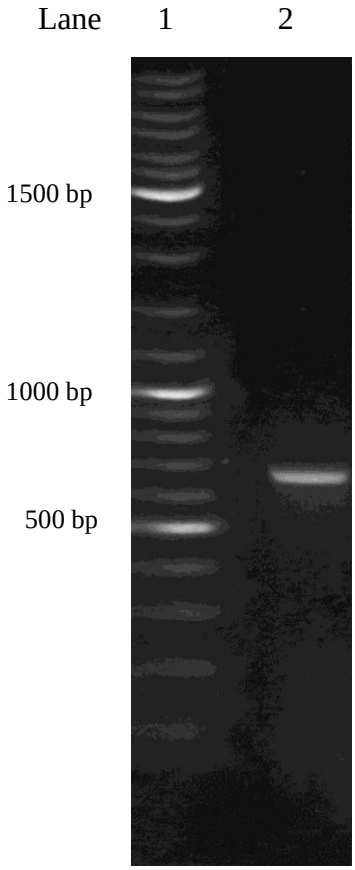


Fig 6.3 Gel Image of RT-PCR Product of *LcyE* Gene Amplification. RT-PCR results with *LcyE* primers. GeneRuler DNA Ladder Mix (Fermentas) is in lane 1 and the RT-PCR product is in lane 2.

6.3.2.2 Complementary DNA (cDNA) Sequence of LcyE

The cDNA sequence for the *LcyE* gene was obtained after the RT-PCR process. cDNA sequence with 618 nucleotides were obtained and is shown in Figure 6.4.

```
1   CTTGAGANGANNNCTATTCTTATTGGCCGTGCTTATGGACGTGTTAGTCG
51  CCATTTGCCCATGAGGAGTTGTTGAAAAGGTGTTTGGAGTCAGGGGTTTC
101 ATATCTTAACTTGAAAGTGGAAAGGATTGTTGAGAATGCAATTGGTCAGA
151 GTCTTGTGGAATGTGAAGGCAATGTCATCATTCCATGCAGGCTAGTTACT
201 GTTGCATCTGGAGCTGCCTCAGGGAAATTGTTGCAGTATGAGGTTGGGGG
251 TCCAAGGGTTTCTGTTCAAACAGCTTATGGTGTGGAGGTTGAGGTGGAAA
301 ACAATCCATATGACCCCAGCCTGATGGTTTTCATGGATTACAGAGACTAT
351 GTCAGACACAAAGTTCACTCTTTAGAAGCAGAATTTCCAACCTTTCTTTA
401 CGCAATGCCCATGTCTGATACAAGAGTCTTTTTTGAGGAAACTTGTTTGG
451 CTTCAAAAGCAGCTATGCCTTTTGATCTCTTGAACAAGAAACTAACGGCA
501 AGACTGGAGACAATGGGTATCCGAATAAAGAAAATTTATGATCAGGAATG
551 GTCTTATATACCACTTGGTGGATCATTGCCAAATACTGATCAAAGAAACC
601 TTGCTTTTCCCGCTGCTG
```

Fig 6.4 cDNA sequence for *LcyE*

6.3.2.3 Translated Amino Acid Sequence

cDNA sequence was translated to amino acid sequence using conversion tool provided in PROSITE. The translated amino acid sequence is shown in Figure 6.5.

```
YSYWPCLWTCSPFAPGVVKRCLESGVSYLNLKVERIVENAIGQSLVECEG
NVIIPCRLVTVASGAASGKLLQYEVGGPRVSVQTAYGVEVEVENNPYDPS
LMVFMDYRDYVRHKVHSLEAEFPTFLYAMPMSDTRVFFEETCLASKAAMP
FDLLNKKLTARLETMGIRIKKIYDQEWSYIPLGGSLPNTDQRNLAFPAA
```

Fig 6.5 Translated amino acid sequence for *LcyE*

6.3.2.4 Sequence Homology Using BLASTp

BLASTp homology search for *LcyE* sequence showed the following matches in the database:

1. 91% homology with *Ipomoea* sp. *Kenyan* lycopene epsilon cyclase (a)
2. 91% homology with *Coffea canephora* lycopene epsilon cyclase (b)
3. 91% homology with *Camellia sinensis* var. assamica lycopene epsilon cyclase (c)
4. 91% homology with *Ricinus communis* lycopene epsilon cyclase, putative chloroplast precursor (d)

Accession numbers for protein sequences with high homology

(a) Accession number BA147576.1 – Yamamizo *et al*., 2010

(b) Accession number ABC87738.1– Sim Kin *et al*., 2008

(c) Accession number ADK74376.1– Borchetia *et al*., 2011

(d) Accession number XP_002514136.1– Chan *et al*., 2009

6.3.2.5 Signature Pattern

ScanProsite analysis strongly suggests that the *LcyE* belongs to the lycopene cyclase protein family. It also has several signature patterns as shown in Table 6.3.

Table 6.3 Signature Patterns for *LcyE* Amino Acids Sequence

Signature Pattern	No. of sites	Location Site
Casein kinase II phosphorylation	1	S_{44}-E_{47}
N-myristoylation	2	G_{64}-K_{69}, G_{184}-T_{189}
Protein kinase C phosphorylation	2	S_{67}-K_{69}, T_{159}-R_{161}
cAMP- and cGMP-dependent protein kinase phosphorylation	1	K_{156}-T_{159}

6.3.2.6 Analysis of Amino Acid Sequence using ProtParam

ProtParam analysis of the *LcyE* amino acid sequence is shown in Table 6.4.

Table 6.4 Physico-Chemical Properties of *LcyE* Amino Acids Sequence

Physico-chemical properties	*LcyE*
Molecular Weight	22.4 kDa
Theoritical pI	5.49
Major Amino Acid Composition	Leu (L) 9.5% Val (V) 9.5% Ala (A) 7.5%
Instability index	39.41 (Classified as stable)

6.3.2.7 Multiple Sequence Alignment for *LcyE* Using ClustalW

Multiple sequence alignment between *I. batatas* sequence and *C. canephora*, *I. kenyan*, *C. sinensis* and *R. communis* sequences is presented in Figure 6.6.

```
gi|C.canephora|      ILIG-RAYGRFSRHLLHEELLRRCVESGVSYLSSKVERIVEAATGHSLVECEGSIVIPCR 95
gi|I.kenyan|         ILIG-RAYGRVSRHLLHEELLRRCLESGVSYLNLKVERIVENAIGQSLVECEGNVIIPCR 118
gi|C.sinensis|       FLIGPCFIGRVSRYLLHEELVKRCVESGVSYLSSKVERIIESAIGHSLIECEQNVVVPCR 240
gi|Sample|           FAPG---------------VVKRCLESGVSYLNLKVERIVENAIGQSLVECEGNVIIPCR 57
gi|R.communis|       ILIG-RAYGRVNRHLLHEELLRRCVESGVSYLSSKVERIIEAADGHSLVACEHDVVVSCR 232
                     :  *               :::**:*******. *****:* * *:**: ** .::::.**

gi|C.canephora|      LATVASGAASGKLLQYELGGPRVSVQTAYGVEVEVENNPYDPNLMVFMDYRDYMRGKVES 155
gi|I.kenyan|         LVTVASGAALGKLLQYEVGGPRVSVQTAYGVEVEVENNPYDPSLMVFMDYRDYVRHKVHS 178
gi|C.sinensis|       LATVASGAASGKLLQYEVGGPRVSVQTAYGMEVEVENNPYDPNLMVFMDYRDYVKPRVQC 300
gi|Sample|           LVTVASGAASGKLLQYEVGGPRVSVQTAYGVEVEVENNPYDPSLMVFMDYRDYVRHKVHS 117
gi|R.communis|       LATVASGAASGKLLQYEVGGPRVSVQTAYGVEVEVENNPYDPSLMVFMDYRDYIKQKVPH 292
                     *.******* *******:************:***********.**********:: :*

gi|C.canephora|      LEAEFPTFLYAMPMSPTRVFFEETCLASKDAMPFELLKKKLMSRLDTLG----VRIIKTY 211
gi|I.kenyan|         LEAEFPTFLYAMPMSDTRVFFEETCLVSKEAMPFDLLKNKLMLRLKTGGDNGYPNKEKFM 238
gi|C.sinensis|       LEAQYPTFLYAMPMSPTRVFFEETCLASKDAMPFDLLKKKLMSRLETMG----VRVIKTY 356
gi|Sample|           LEAEFPTFLYAMPMSDTRVFFEETCLASKAAMPFDLLNKKLTARLETMG----IRIKKIY 173
gi|R.communis|       LEAEYPTFLYAMPMSSTRVFFEETCLASKGAMPFDLLKRKLMSRLETMG----IRILKTY 348
                     ***::********** **********.** ****:**:.**  **.* *     .  *

gi|C.canephora|      EEEWSYIPVGGSLPNTEQKNLAFGAAASMVHPATGYSVVRSLSEAPKYASAIANILKQGQ 271
gi|I.kenyan|         KRNGLIYQLVDPCQILIKRNLAFGAAASMVHPATGYSVVRSLSEAPRYASVIANILKRSP 298
gi|C.sinensis|       EEEWSYIPVGGSLPNTEQKNLALGAAASMVHPATGYFVVRSLSEAPKYASVIANILKQGH 416
gi|Sample|           DQEWSYIPLGGSLPNTDQRNLAFPAA-------------------------------- 199
gi|R.communis|       EEEWSYIPVGGSLPNTEQKNLAFGAAASMVHPATGYSVVRSLSEAPNYASVIASILKQDQ 408
                     ..:     : ..     ::***: **
```

Fig 6.6 Multiple sequence alignment for *I. batatas LcyE* amino acid sequence with *C. canephora, I. kenyan, C. sinensis* and *R. communis*

6.3.2.8 Phylogenetic Tree for *LcyE*

Phylogenetic tree for the *LcyE* sequence (Figure 6.7) was generated from ClustalW. The putative *LcyE* sequence from *I. batatas* was placed in the same cluster as the *R. communis* and both share a same cluster with *C. sinensis*. However, the *I. kenyan* was located in a separated cluster from the *LcyE*.

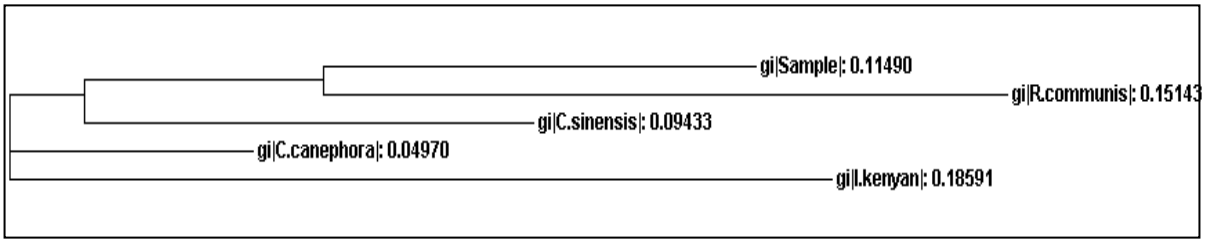


Fig 6.7 Phylogeny tree showing the evolutionary relationship and distance between the *I. batatas LcyE* amino acid sequence and other sequences

6.3.2.9 Protein Structure Prediction

The *LcyE* structure was predicted using Swiss-Prot software and viewed using Rasmol software. The red and blue ribbons indicate the end terminals of the proteins. It was predicted that this protein contained 92 hydrogen bonds, 3 helices, 11 strands and 13 turns. Figure 6.8 shows the predicted structure of the LcyE protein.

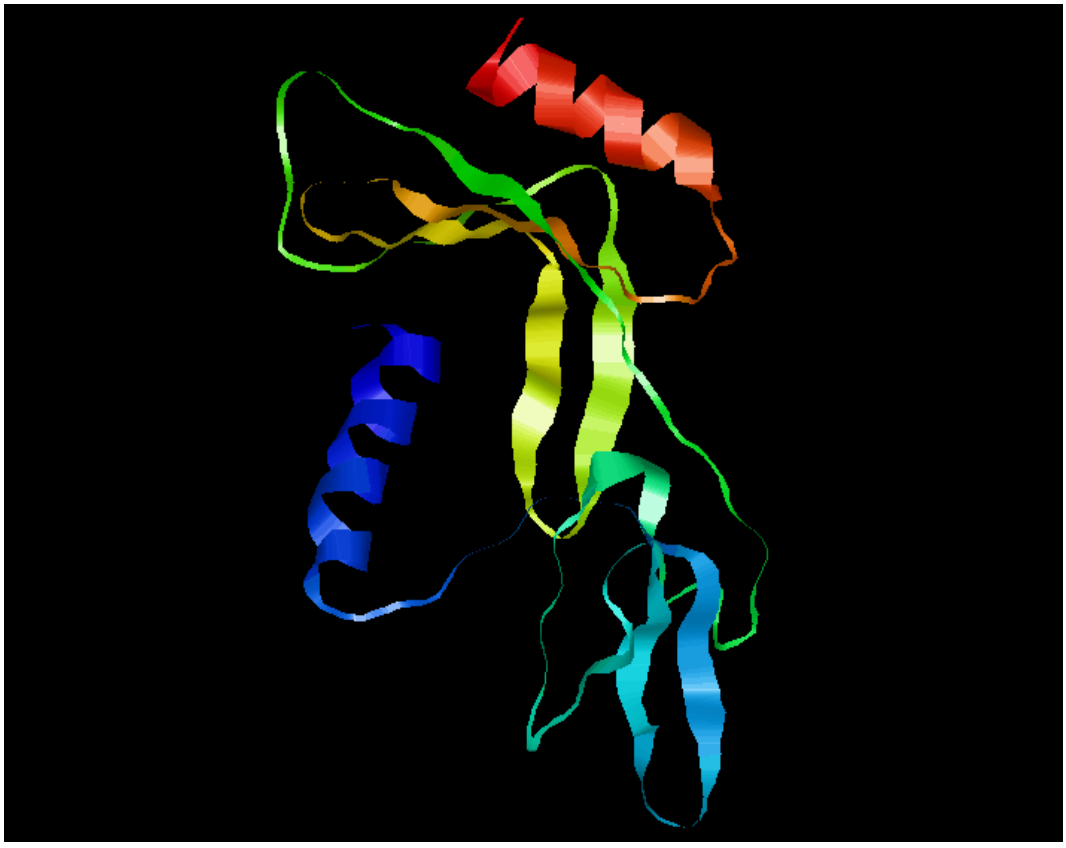


Fig 6.8 Predicted protein structure of *LcyE* from *I. batatas*

6.3.3 *LcyB* Gene

6.3.3.1 Agarose Gel Electrophoresis

RT-PCR with the *LcyB* primers yielded a single product with a size between 850 bp to 900 bp as shown in Figure 6.9.

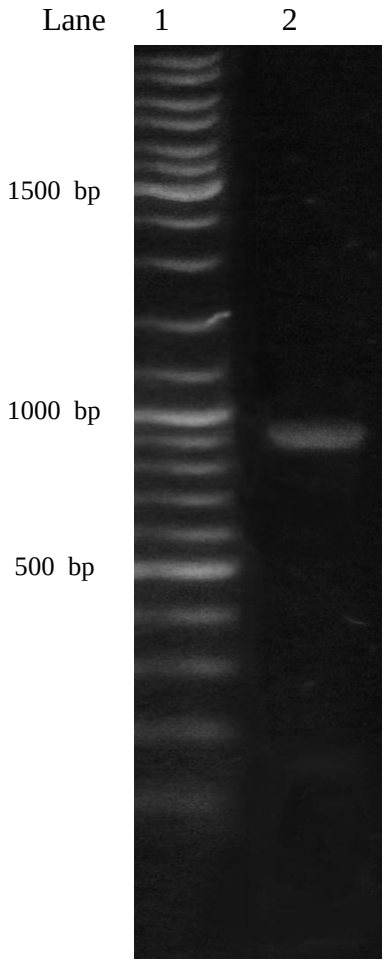


Fig 6.9 Gel Image of RT-PCR Product of *LcyB* Gene Amplification. RT-PCR results with *LcyB* primers. GeneRuler DNA Ladder Mix (Fermentas) is in lane 1 and the RT-PCR product is in lane 2.

6.3.3.2 Complementary DNA (cDNA) Sequence of *LcyB*

The cDNA sequence for the *LcyB* gene was obtained after the RT-PCR process. cDNA sequence with 894 nucleotides were obtained and is shown in Figure 6.10.

```
1   CCCCCTTTGAAACCCAAAAAAGGGAAATTAGGATTGGTTCATTGAAACCT
51  TGCAGGAATGTTGGCCGTGTTAAGGCCAGCAGTAGTGCCCTATTAGAACT
101 TGTGCCTGTGACCAAGAAAGAGAATCTTGATTTTGAGCTTCCTATGTTTG
151 AACCCTCTAAAGGGATTGTTGTGGATTTAGCTGTGGTTGGGGGTGGCCCT
201 GCTGGGCTTGCAGTGGCTCAGCAGGTTTCACAAGCTGGGTTATCAGTTTG
251 TTCAATTGACCCCTCTCCCAAATTGATTTGGCCCAATAATTATGGGGTTT
301 GGGTGGATGAATTTGAGGCCATGGATTTGTTGGATTGCCTCGATACCACG
351 TGGTCTGGAGCTATGGTGTATATTGATGACCGCACGACTAAAGATCTTGA
401 CAGGCCTTATGGGCGGGTTAACAGGAAGAAACTCAAATCCAAAATGATGC
451 AGAAATGCATTGCCAATGGTGTTAAGTTTCATCAAGCCAAGGTTATAAAG
501 GTGATCCATGAAGAATCGAAATCCATGTTGATTTGCAGTGACGGTGTGAC
551 TATTCAAGCAACTGTGGTTCTTGATGCAACTGGCTTTTCTAGATGCCTAG
601 TTCAGTACGATAAGCCTTATAATCCGGGCTATCAAGTTGCGTATGGCATT
651 CTTGCAGAAGTGGAGGAACACCCTTATGATTTGAATAAGATGGTTTTCAT
701 GGATTGGCGAGACTCTCACCTAAACAGTAACTTGGAGCTAAAGGAGAGAA
751 ATAAAAGAATCCCCGACTTTTCTTTATGCCCATGCCCATTTTCCTCGCAA
801 GAGGATATTCCTTTGAAGAAAACCTCCACTTAGTGGCTTCGTCCTTGGCT
851 TTGAATATTGAAAGGATATTTCAAGGAAAAGAAATGGTTGGTTT
```

Fig 6.10 cDNA sequence for *LcyB*

6.3.3.3 Translated Amino Acid Sequence

cDNA sequence was translated to amino acid sequence using conversion tool provided in PROSITE. The translated amino acid sequence was shown in Figure 6.11 below.

```
PFETQKREIRIGSLKPCRNVGRVKASSSALLELVPVTKKENLDFELPMFE
PSKGIVVDLAVVGGGPAGLAVAQQVSQAGLSVCSIDPSPKLIWPNNYGVW
VDEFEAMDLLDCLDTTWSGAMVYIDDRTTKDLDRPYGRVNRKKLKSKMMQ
KCIANGVKFHQAKVIKVIHEESKSMLICSDGVTIQATVVLDATGFSRCLV
QYDKPYNPGYQVAYGILAEVEEHPYDLNKMVFMDWRDSHLNSNLELKERN
KRIPDFSLCPCPFSSQEDIPLKKTSTWLRPWLILKGYFKEKKWLV
```

Fig 6.11 Translated amino acid sequence for *LcyB*

6.3.3.4 Sequence Homology Using BLASTp

BLASTp homology search on *LcyB* revealed the following matches in the database:

1. 95% homology with *Ipomoea* sp. *Kenyan* lycopene β-cyclase (a)

2. 95% homology with *Solanum lycopersicum* chloroplastic lycopene β-cyclase(b)

3. 95% homology with *Citrus sinensis*, lycopene β-cyclase (c)

4. 94% homology with *Carica papaya*, lycopene β-cyclase (d)

Accession numbers for protein sequences with high homology

(a) Accession number BAI47577.1 – Yamamizo *et al*., 2010

(b) Accession number NP_001234226.1 - Wan *et al*., 2007

(c) Accession number AAU05145.1 - Tao *et al*., 2004

(d) Accession number ABD91578.1 – Skelton *et al*., 2006

6.3.3.5 Signature Pattern

ScanProsite analysis strongly suggests that the *LcyB* belongs to the lycopene cyclase protein family. It also has several signature patterns as shown in Table 6.5.

Table 6.5 Signature Patterns of *LcyB* Amino Acids Sequence

Signature Pattern	No. of sites	Location Site
Protein kinase C phosphorylation	5	T_4-K_6, S_{13}-K_{15}, T_{37}-K_{39}, S_{88}-K_{90}, T_{128}-K_{130}
Casein kinase II phosphorylation	5	T_{37}-E_{40}, T_{128}-D_{131}, S_{242}-E_{245}, S_{264}-E_{267}, S_{265}-D_{268}
N-myristoylation	4	G_{63}-G_{68}, G_{64}-L_{69}, G_{68}-Q_{73}, G_{79}-S_{84}
Tyrosine kinase phosphorylation	1	K_{130}-Y_{136}
cAMP- and cGMP-dependent protein kinase phosphorylation	1	K_{272}-S_{275}

6.3.3.6 Analysis of Amino Acid Sequence using ProtParam

ProtParam analysis of the *LcyB* amino acid sequence is shown in Table 6.6.

Table 6.6 Physico-Chemical Properties of *LcyB* Amino Acids Sequence

Physico-chemical properties	*LcyB*
Molecular Weight	33.4 kDa
Theoritical pI	8.56
Major Amino Acid Composition	Leu (L) 9.8% Lys (K) 9.2% Ser (S) 7.1%
Instability index	27.48 (Classified as stable)

6.3.3.7 Multiple Sequence Alignment for *LcyB* Using ClustalW

Multiple sequence alignment between *I. batatas* sequence and *I. kenyan*, *C. sinensis*, *S. lycopersicum* and *C. papaya* sequences are presented in Figure 6.12.

```
gi|C.sinensis|          MDTLLKTHNKLEFLPQVHGALEKSSSLSSLKIQNQELRFGLKKSRQKRNRSCFIKASSSA 60
gi|C.papaya|            MDTLLKTHNKLEFLPQLHGFSEKSSSSSS-RLQNPELRFGPKKYPWKRGRDGCLKASNSA 59
gi|S.lycopersicum|      MDTLLKTPNNLEFLNPHHGFAVKASTFRSEKHHN----FGSRKFCETLGRSVCVKGSSSA 56
gi|I.Kenyan|            MDTLLKTPNKLEFLHPHHGFAVKASAFTSLKPQKQEIRIGSWKACRNVGR---VKASSSA 57
gi|Sample|              ----------------------------PFETQKREIRIGSLKPCRNVGR---VKASSSA 29
                                                  . . ::    :*  *   . .*   :*.*.**

gi|C.sinensis|          LLELVPETKKENLEFELPMYDPSKGLVVDLAVVGGGPAGLAVAQQVSAAGLSVCSIDPSP 120
gi|C.papaya|            LLELVPETKKETLDFELPMYDPSKGLVVDLAVVGGGPAGLAVAQQVSQAGLSVCSIDPSP 119
gi|S.lycopersicum|      LLELVPETKKENLDFELPMYDPSKGVVVDLAVVGGGPAGLAVAQQVSEAGLSVCSIDPNP 116
gi|I.Kenyan|            LLELVPVTKKENLDFELPMFEPSKGIVVDLAVVGGGPAGLAVAQQVSQAGLSVCSIDPSP 117
gi|Sample|              LLELVPVTKKENLDFELPMFEPSKGIVVDLAVVGGGPAGLAVAQQVSQAGLSVCSIDPSP 89
                        ****** ****.*:*****::****:********************** **********.*

gi|C.sinensis|          KLIWPNNYGVWVDEFEAMDLLDCLDTTWSGAVVHIDDNTKKDLDRPYGRVNRKLLKSKML 180
gi|C.papaya|            KLIWPNNYGVWVDEFEAMDLLDCLDTTWSGAVVYIDDKSKKDLDRPYGRVNRKQLKSKML 179
gi|S.lycopersicum|      KLIWPNNYGVWVDEFEAMDLLDCLDATWSGAAVYIDDNTAKDLHRPYGRVNRKQLKSKMM 176
gi|I.Kenyan|            KLIWPNNYGVWVDEFEAMDLLDCLDTTWSGAMVYIDDRTTKDLDRPYGRVNRKKLKSKMM 177
gi|Sample|              KLIWPNNYGVWVDEFEAMDLLDCLDTTWSGAMVYIDDRTTKDLDRPYGRVNRKKLKSKMM 149
                        *************************:***** *:***.: ***.********* *****:

gi|C.sinensis|          QKCITNGVKFHQAKVIKVIHEESKSLLICNDGVTIQAAVVLDATGFSRCLVQYDKPYNPG 240
gi|C.papaya|            QKCIANGVKFHQAKVIKAIHEESKSLLICNDGVTIQATVVLDATGFSRCLVQYDRPYNPG 239
gi|S.lycopersicum|      QKCIMNGVKFHQAKVIKVIHEESKSMLICNDGITIQATVVLDATGFSRSLVQYDKPYNPG 236
gi|I.Kenyan|            QKCIANGVKFHQAKVIKVIHEESKSMLICSDGVTIQATVVLDATGFSRCLVQYNKPYNPG 237
gi|Sample|              QKCIANGVKFHQAKVIKVIHEESKSMLICSDGVTIQATVVLDATGFSRCLVQYDKPYNPG 209
                        **** ************.*******:***.**:****:**********.****::*****

gi|C.sinensis|          YQVAYGILAEVEEHPFDLDKMVFMDWRDSHLNNNSELKEANSKIPTF-LYAMPFSSNRIF 299
gi|C.papaya|            YQVAYGILAEVEKHPFDVNKMVFMDWRDSHLNSNTELKERNSRIPTF-LYAMPFSSNRIF 298
gi|S.lycopersicum|      YQVAYGILAEVEEHPFDVNKMVFMDWRDSHLKNNTDLKERNSRIPTF-LYAMPFSSNRIF 295
gi|I.Kenyan|            YQVAYGILAEVEKHPYDLNKMVFMDWRDSHLNSNLELKERNKRIPTF-LYAMPFSSNRIF 296
gi|Sample|              YQVAYGILAEVEEHPYDLNKMVFMDWRDSHLNSNLELKERNKRIPDFSLCPCPFSS---- 265
                        ************:**:*::************:.* :*** *.:** * * . ****

gi|C.sinensis|          LEETSLVARPGVPMKDIQERMVARLKHLGIKVRGIEEDEHCVIPMGGPLPVLPQRVVGIG 359
gi|C.papaya|            LEETSLVARPGLRMEDIQERMVARLTHLGVKVISIEEDEHCVIPMGGPLPVLPQRVIGIG 358
gi|S.lycopersicum|      LEETSLVARPGLRIDDIQERMVARLNHLGIKVKSIEEDEHCLIPMGGPLPVLPQRVVGIG 355
gi|I.Kenyan|            LEETSLGARPGLDMKDIQERMGARLRHLGINVKSIEEDERCVIPMGGPLPVIPQRVVGIG 356
gi|Sample|              -----------------------------------QED----IPLK-------------- 272
                                                          :**    **:
```

Fig 6.12 Multiple sequence alignment for *I. batatas LcyB* amino acids sequence with *I. kenyan, C. sinensis, S. lycopersicum* and *C. papaya*

6.3.3.8 Phylogenetic Tree for *LcyB*

Phylogenetic tree for *LcyB* was constructed from the ClustalW analysis. The *LcyB* sequence from *I. batatas* was located in the same cluster as the *I. Kenyan* and this showed a close phylogeny relationship between the two sequences. *S. lycopersicum, C. sinensis* and *C. papaya* were located in separate cluster.

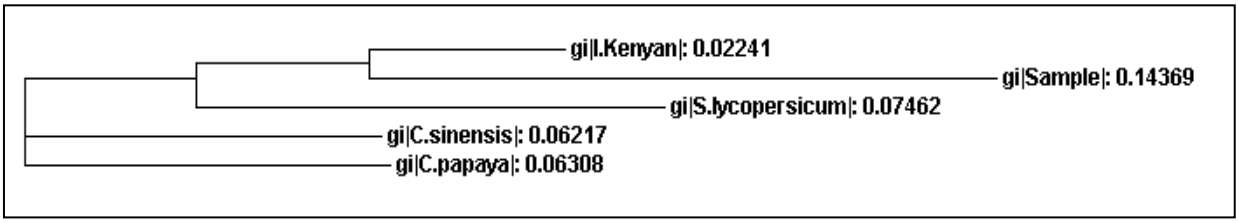


Fig 6.13 Phylogeny tree showing the evolutionary relationship and distance between the *I. batatas LcyB* amino acid sequence and other sequences

6.3.3.9 Protein Structure Prediction

The *LcyB* structure was predicted using Swiss-Prot software and viewed using Rasmol software. The red and blue ribbons indicate the end terminals of the protein. This putative protein structure was predicted to have 129 hydrogen bonds, 6 helices, 21 strands and 20 turns. Figure 6.14 shows the predicted structure of the *LcyB* protein.

Fig 6.14 Predicted protein structure of *LcyB* from *I. batatas*

6.3.4 *Psy* Gene

6.3.2.1 Agarose Gel Electrophoresis

RT-PCR with the *Psy* primers yielded a single product with a size between 950 bp to 1000 bp as shown in Figure 6.15.

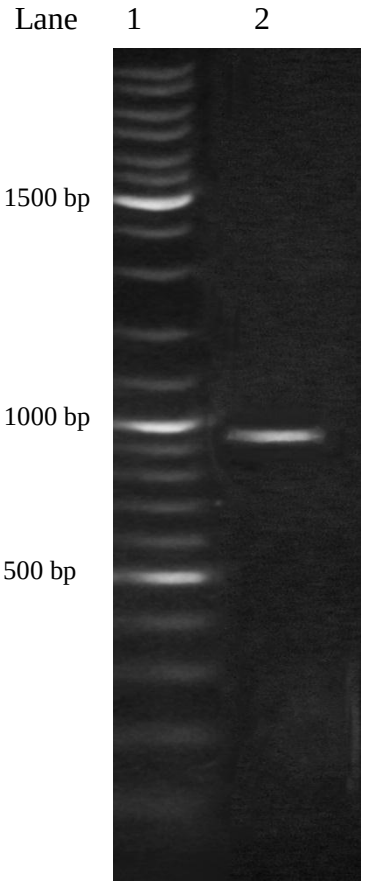


Fig 6.15 Gel Image of RT-PCR Product of Psy Gene Amplification. RT-PCR results with *Psy* primers. GeneRuler DNA Ladder Mix (Fermentas) is in lane 1 and the RT-PCR product is in lane 2.

6.3.4.2 Complementary DNA (cDNA) Sequence of Psy

The cDNA sequence for the *Psy* gene was obtained after the RT-PCR process. cDNA sequence with 956 nucleotides were obtained and is shown in Figure 6.16.

```
1   GGTTTAAGCGGGGAATTGTGCAGAGTATGCTAAGACGTTTTATTTGGGAA
51  CCATGCTAATGACACCTGAGAGAAGAAGAGCTATCTGGGCGATATATGTG
101 TGGTGTAGGAGAACTGATGAGCTCGTTGACGGGCCTAATGCATCGCATAT
151 AACTCCAACCGCCCTGGATAGATGGGAGGCTCGGCTGGAAGACGTATTCA
201 GAGGGCGCCCGTTTGATATGCTCGACGCTGCACTATCAGATACAGTATCC
251 AGGTTTCCAGTTGATATTCAGCCCTTTAGGGATATGATTGAAGGAATGCG
301 AATGGACCTCTGGAAATCGAGATACGATAACTTTGATGAGCTATATCTGT
351 ACTGTTATTACGTTGCTGGTACAGTTGGTTTGATGAGTGTCCCGGTTATG
401 GGCATTGCGCCCGAATCAAAGGCAACTACAGAGAGTGTCTATAATGCCGC
451 TTTGGCTTTAGGCATCGCTAATCAACTAACCAACATTCTCAGAGACGTAG
501 GCGAGGATGCTAGACGGGGGAGGGTCTATTTACCTCAAGATGAATTAGCT
551 CAAGCGGGACTCTCTGATGACGATATATATGCTGGAAAAGTTACTGATAA
601 GTGGAGGAACTTCATGAAGAAGCAAATCAAGAGAGCAAGGAAGTTCTTCG
651 ACGAGGCTGAGAGAGGCGTGACTGAACTTAGCTCCGCTAGTCGATGGCCA
701 GTGTGGGCGTCGCTGCTGTTGTACCGCAAGATACTCGACGAGATCGAAGC
751 CAACGACTACAACAACTTCACAAGGAGAGCCTATGTAAGCAAGCCAAAGA
801 AACTGCTTGCATTGCCTATTGCATATGCAAAAGCTGTGATTCGACCATCA
851 ACAACTACTTTCACCTCATCGATCAATAAGCTTCACTCAACATCCCCCAC
901 TAATTCTTATGTGGCGCCGCCGGTTCCTTTCAATATCTTCATTGTAAATC
951 ACGTTC
```

Fig 6.16 cDNA sequence for *Psy*

6.3.4.3 Translated Amino Acid Sequence

cDNA sequence was translated to amino acid sequence using conversion tool provided in PROSITE. The translated amino acid sequence was shown in Figure 6.17 below.

```
VAGNCAEYAKTFYLGTMLMTPERRRAIWAIYVWCRRTDELVDGPNASHIT
PTALDRWEARLEDVFRGRPFDMLDAALSDTVSRFPVDIQPFRDMIEGMRM
DLWKSRYDNFDELYLYCYYVAGTVGLMSVPVMGIAPESKATTESVYNAAL
ALGIANQLTNILRDVGEDARRGRVYLPQDELAQAGLSDDDIYAGKVTDKW
RNFMKKQIKRARKFFDEAERGVTELSSASRWPVWASLLLYRKILDEIEAN
DYNNFTRRAYVSKPKKLLALPIAYAKAVIRPSTTTFTSSINKLHSTSPTN
SYVAPPVPFNIFIVNHV
```

Fig 6.17 Translated amino acids sequence for *Psy*

6.3.4.4 Sequence Homology Using BLASTp

BLASTp homology search on *Psy* revealed the following matches in the database:

(1) 91% homology with *Prunus mume* phytoene synthase (a)

(2) 91% homology with *Carica papaya* phytoene synthase (b)

(3) 90% homology with *Actinidia deliciosa* phytoene synthase (c)

(4) 88% homology with *Ipomoea* sp. *Kenyan* phytoene synthase (d)

Accession numbers for protein sequences with high homology

(a) Accession number BAF49052.1- Kita *et al*., 2007

(b) Accession number ABG72805.1 – Liao and Chen, 2006

(c) Accession number ACO53104.1 - Ampomah-Dwamena *et al*., 2009

(d) Accession number BAI47572 - Yamamizo *et al*., 2010

6.3.4.5 Signature Pattern

ScanProsite analysis strongly suggests that the *Psy* belongs to the squalene/phytoene synthase protein domain. It also has several signature patterns as shown in Table 6.7.

Table 6.7 Signature Patterns for *Psy* Amino Acids Sequence

Signature Pattern	No. of Sites	Location Site
Squalene and phytoene synthases signature 1 (YCyyVAGTVGlmSvpV)	1	Y_{116}-V_{131}
Squalene and phytoene synthase signature 2 (LGianQlt.NIlRDVgeDar rg...RvYlP)	1	L_{152}-P_{177}
N-glycosylation	2	N_{45}-H_{48}, N_{254}-R_{257}
Casein kinase II phosphorylation	3	T_{52}-D_{55}, S_{105}-D_{108}, S_{187}-D_{190}
Tyrosine kinase phosphorylation	1	K_{139}-Y_{146}
Protein kinase C phosphorylation	2	T_{197}-K_{199}, T_{256}-R_{258}
Microbodies C-terminal targeting signal	1	N_{315}-V_{317}

6.3.4.6 Analysis of Amino Acid Sequence Using ProtParam

ProtParam analysis of the *Psy* amino acid sequence is shown in Table 6.8.

Table 6.8 Physico-Chemical Properties of *Psy* Amino Acids Sequence

Physico-chemical properties	*Psy*
Molecular Weight	36.1 kDa
Theoritical pI	8.61
Major Amino Acid Composition	Ala (A) 10.1% Leu (L) 8.5% Arg (R) 8.2%
Instability index	52.58 (Classified as unstable)

6.3.4.7 Multiple Sequence Alignment for *Psy* Using ClustalW

Multiple sequence alignment between *I. batatas* sequence and *P. mume, A. deliciosa, C. papaya* and *I. Kenyan* sequences are presented in Figure 6.18.

```
gi|I.Kenyan|        KLRSRD-DLEVKPDIVLPGDLGVLSEAYDRCGEVCAEYAKTFYLGTMLMTPERRRAIWAI 178
gi|Sample|          ----------------------------VAGNCAEYAKTFYLGTMLMTPERRRAIWAI 30
gi|C.papaya|        QLRSSSGELDVKPDIVLPGTLSLMTEAYDRCGEVCAEYAKTFYLGTLLMTPERRKAIWAI 175
gi|P.mume|          QLKSNG-DLD-EPDIVLPGNLSLMSDAYDRCGEVCAEYAKTFYLGTLLMTPERRRAIWAI 163
gi|A.deliciosa|     QLETGQ-DYDVKPDIVLPGTLSLLGEAYDRCGEVCAEYAKTFYLGTMLMTPERRKAIWAI 175
                                                .  ************:*******:*****

gi|I.Kenyan|        YVWCRRTDELVDGPNASHITPTALDRWEAMLEDVFRGRPFDMLDAALSDTVSRFPVDIQP 238
gi|Sample|          YVWCRRTDELVDGPNASHITPTALDRWEARLEDVFRGRPFDMLDAALSDTVSRFPVDIQP 90
gi|C.papaya|        YVWWRRTEELVDGPNASHITPTALDRWEARLEDLFQGRPFDMLDAALSDTVAKFPVDIQP 235
gi|P.mume|          YVWCRRTDELVDGPNASHITPTALDRWESRLEDLFQGRPFDMLDAALSDTVNKFPVDIQP 223
gi|A.deliciosa|     YVWCRRTDELVDGPNASHITPTALDRWESRLEDIFLGRPFDMLDAALSDTVTKFPVDIQP 235
                    *** ***:*********************: ***:* *************** :*******

gi|I.Kenyan|        FRDMIEGMRMDLWKSRYDNFDELYLYCYYVAGTVGLMSVPVMGIAPESKATTESVYNAAL 298
gi|Sample|          FRDMIEGMRMDLWKSRYDNFDELYLYCYYVAGTVGLMSVPVMGIAPESKATTESVYNAAL 150
gi|C.papaya|        FIDMIEGMRMDLRKSRYKNFDELYLYCYYVAGTVGLMSVPIMGIAPESQATTESVYNAAL 295
gi|P.mume|          FKDMIEGMRMDLRKSIYQNFDELYLYCYYVAGTVGLMSVPVMGISPESQATTESVYNAAL 283
gi|A.deliciosa|     FKDMIEGMRMDLRKSRYKNFDELYLYCYYVAGTVGLMSVPIMGIAPDSLATTESVYNAAL 295
                    * ********** ** *.**********************:***:*:* ***********

gi|I.Kenyan|        ALGIANQLTNILRDVGEDARRGRVYLPQDELAQAGLCDEDIYAGKVTDKWRNFMKKQIKR 358
gi|Sample|          ALGIANQLTNILRDVGEDARRGRVYLPQDELAQAGLSDDDIYAGKVTDKWRNFMKKQIKR 210
gi|C.papaya|        ALGIANQLTNILRDVGEDARRGRIYLPQDELAQAGLSDDDIFAGTVTDKWRNFMKKQIKR 355
gi|P.mume|          ALGIANQLTNILRDVGEDARRGRIYLPQDELAQAGLSDSDIYAGKVTDKWRSFMKNQIKR 343
gi|A.deliciosa|     ALGIANQLTNILRDVGEDARRGRVYLPQDELAQAGLSDEDIFAGKVTDKWRIFMKHQIKR 355
                    **********************:************.*.**:**.****** ***:****

gi|I.Kenyan|        ARKFFDEAERGVTELGSASRWPVWASLLLYRKILDEIEANDYNNFTRRAYVSKPKKLLAL 418
gi|Sample|          ARKFFDEAERGVTELSSASRWPVWASLLLYRKILDEIEANDYNNFTRRAYVSKPKKLLAL 270
gi|C.papaya|        ARMFFDEAEKGVTELSAASRWPVWASLLLYRKILDEIEANDYNNFTKRAYVSKAKKILAL 415
gi|P.mume|          ARMFFDEAEKGVTELSEASRWPVWASLLLYRQILDEIEANDYNNFTRRAYVSKAKKLLAL 403
gi|A.deliciosa|     ARMFFDEAEKGVTELSSASRWPVWASLLLYRQILDEIEANDYNNFTRRAYVSKPKKVLAL 415
                    ** ******:*****. **************:***************:******.**:***

gi|I.Kenyan|        PIAYAKALIRPSTT--TSPLVKA------------------------ 439
gi|Sample|          PIAYAKAVIRPSTTTFTSSINKLHSTSPTNSYVAPPVPFNIFIVNHV 317
gi|C.papaya|        PISYARSLIGPSRT--SSSANKIQI---------------------- 438
gi|P.mume|          PIAYTKSLIRPSRT--SSYSTRAQKTDSLTSKV-------------- 434
gi|A.deliciosa|     PIAYAKSIVAPTRT--SSLTSVTR----------------------- 437
                    **:*::::: *: *  :*
```

Fig 6.18 Multiple sequence alignment for *I. batatas Psy* amino acid sequence with *P. mume, A. deliciosa, C. papaya* and *I. kenyan*

6.3.4.8 Phylogenetic Tree for *Psy*

Phylogenetic tree for *Psy* sequence was constructed from ClustalW analysis. The *Psy* sequence from the *I. batatas* was located in the same cluster as *I. Kenyan Psy* sequence. *P. mume* and *A. deliciosa* sequences shared the same cluster and showed high homology between them. The *Psy* sequence for *C. papaya* was located at the different cluster.

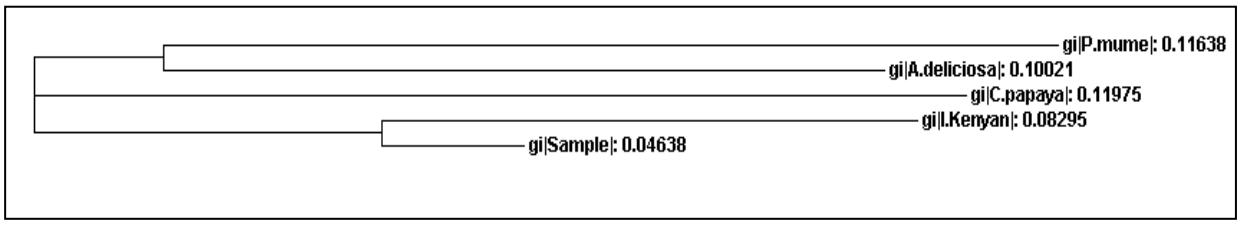


Fig 6.19 Phylogeny tree showing the evolutionary relationship and distance between the *I. batatas Psy* amino acid sequence and other sequences

6.3.4.9 Protein Structure Prediction

The *Psy* structure was predicted using Swiss-Prot software and viewed using Rasmol software. The red and blue ribbons indicate the end terminals of the proteins. It was predicted that this protein contained 196 hydrogen bonds, 17 helices and 19 turns. Figure 6.20 shows the predicted structure of the *Psy* protein

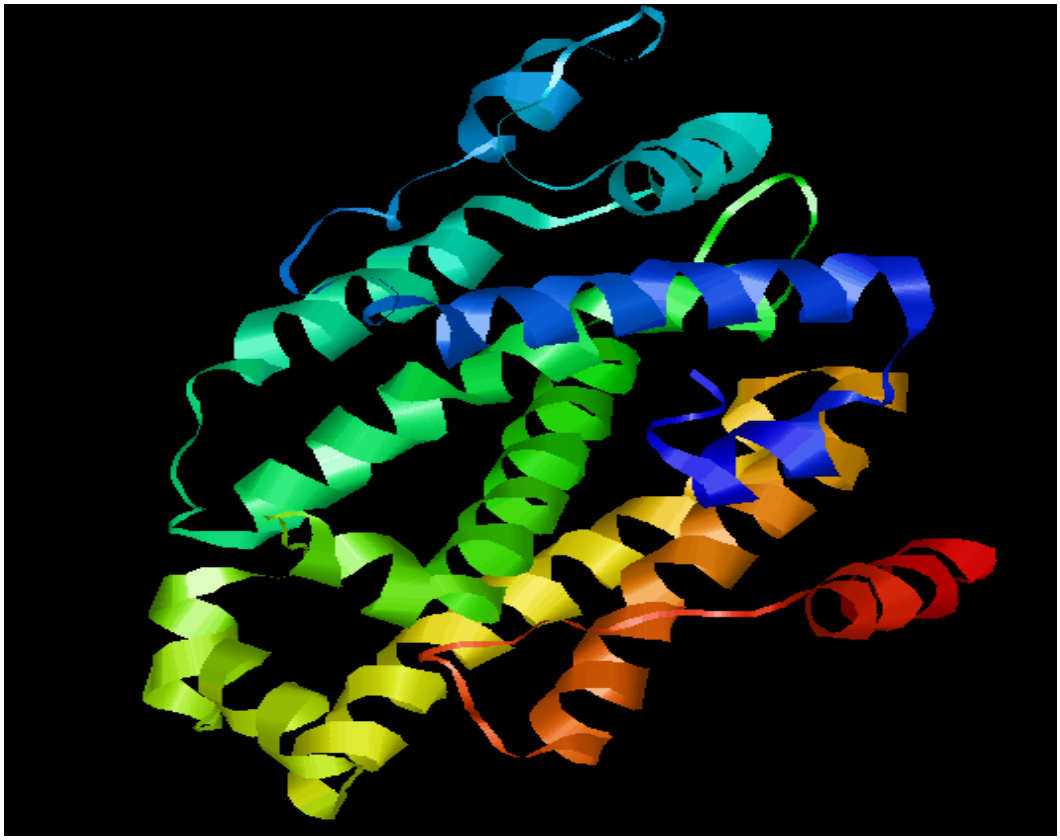


Figure 6.20 Predicted protein structure for *Psy* from *I. batatas*

6.4 DISCUSSION

The study of the molecular aspect of carotenoid biosynthesis pathway in plants allows genetic manipulation of these genes to alter the amount and composition of carotenoids in plants. The first gene cloned was the phytoene desaturase (*Pds*) gene, based on the high similarity between plant and cyanobacteria synthesis pathway (Chamovitz *et al*., 1990). Since then, various methods such as functional complementation, transposon tagging and map-based cloning have been used to isolate different genes in the biosynthesis pathway (Lotan and Hirschberg, 1995; Marin *et al*., 1996). The final carotenoid products from plants are also dependable on the expression of the genes involved in the pathway. The *I. batatas* plant, despite being one of the most highly consumed crops and an important source of β-carotene especially in developing countries, the study of their genes involved in carotenoid biosynthesis cycle are scarce. This study is the first ever isolation and characterisation of the *LcyE*, *LcyB* and *Psy* genes from a Malaysian variety of *I. batatas* leaves. The study on these genes will provide information for future manipulation work to be conducted.

The A_{260}/A_{280} value for the extracted RNA was recorded at 1.85 and thus suitable for downstream processes such as RT-PCR. The isolated *LcyE* sequence recorded total number of 618 nucleotides, as expected from the primers design. The shorter length observed compared to the similar genes from other plants may be due to species variation or plant adaptation to the environment. It was previously suggested that genomic changes were usually observed in highly repetitive fractions of the genome and may be limited to particular area of the chromosomal sites (Cullis and Creissen, 1987). Reorganisation of the genome, particularly observed in response to stress, is believed to be plant adaptation to increase the pool of variability among the species. This is further supported by the observation that *I.* sp. *Kenyan*, which shared the same genus with the *I.*

batatas also contained a lower number of nucleotides in its *LcyE* gene. This sequence is the first *LcyE* gene sequence isolated from the *I. batatas* plant and thus provide new information.

Deduced *LcyE* amino acid sequence was subjected to Blastp and result revealed 91% homology with the *LcyE* sequence form *Coffea canephora*, *Camellia sinensis* var. assamica, *Ricinus communis* and *Ipomoea* sp. *kenyan*. The high percentage of homology observed from the Blastp result confirmed the sequence as a putative *LcyE* sequence. Pfam analysis showed that the *LcyE* gene isolated from *I. batatas* belongs to the lycopene cyclase protein family (PF05834). This family is located under the NADP Rossmann (CL0063) clan and contains two domain proteins. The catalytic domain in this protein family may contribute to the governing role of the *LcyE* gene in the lycopene cyclisation process in carotenoid biosynthesis pathway.

Several signature patterns were observed for the *LcyE* sequence with some overlapping patterns observed in the same sequence region. Overlapping patterns were observed for the N-mrystoylation and protein kinase C phosphorylation site at position 64^{th} to 69^{th} amino acid residues and cAMP- and cGMP-dependent protein kinase phosphorylation and protein kinase C phosphorylation site from the 156^{th} to 161^{th} amino acid residues. Signature databases are important to predict protein function, family identity and in the detection of homologous regions (Attwood and Parry-Smith, 1999). Although the final structure of the protein depends on post-translational modification and alternative splicing of the protein, early predictions may provide substantial information before in-depth analysis is conducted. The shared regions in the *LcyE* may still produce the same individual functions or may result in the overexpression or underexpression of a sequence functions.

Previous studies have reported that the protein kinase C site plays important roles in the activation of cellular functions and proliferation (Kishimoto *et al.*, 1985; Butt *et al*., 1993). Myristoylation process took place at the N-myristoyl site and the conserved N-mrystoylation sequence has been shown to affect the different development aspects in plant (Towler *et al*., 1988; Pierre *et al*., 2007) which may explain the differences observed such as the formation of storage roots in *I. batatas* instead of fruits compared to other plants. Besides that, cyclic GMP was found to function in phytochrome responses and have the ability to trigger anthocyanin production. In the presence of calcium, it can induce the development of fully mature chloroplasts with all the photosynthetic machinery and may modulate the process of gene expression (Bowler *et al*., 1994). The effects of the repetitive patterns in the *LcyE* sequence was unknown of although in yeast, some repetitive signature pattern was suggested to have related function while some other repetitive patterns are found to be false positives for co-regulated genes (Arabnia and Tran, 2011).

Prot Param analysis predicted a stable 22.4 kDa protein with a theoretical pI value of 5.49 from the *LcyE* sequence. The predicted amino acids sequence has the highest composition of leucine and valine at 9.5% and their hydrophobicity may correspond to the membrane spanning helical regions of the protein (Kyte and Doolittle, 1982). A few substitutions, deletions and mutations were observed in the *LcyE* sequence which could possibly due to the different plant species or plant adaptation to the environment stress. Previous study showed that mutation on the single amino acid residue (H457) in the *LcyE* sequence of lettuce was found to contribute the bi-cyclase activity to the enzyme (Cunningham and Gantt, 2001). In maize, mutation in several spots of the *LcyE* sequence was found to contribute to their function both as mono-cyclic and bi-cyclic.

The time and degree of insertion occurrence in a gene sequence may vary among genes and thus may contribute to the different clusters formation in the phylogenetic tree. Previous study found that the absence of tryptophan amino acid in the conserved domain of the *LcyE* sequence in *C. canephora* has resulted in a lower β-carotene and higher α-carotene content, a scenario not commonly observed in other plants' leaves (Zhu *et al*., 2003). The generated phylogenetic tree showed that the *I. batatas* sequence located in the same cluster as the *R. communis* and distantly located from the *I. kenyan* which may denoted high homology between the former pair.

Amplification via RT-PCR yielded the *LcyB* sequence with 894 nucleotides in length, as expected from the LcyBF and LcyBR primers used. The *LcyB* gene was studied due to their function in catalysing the formation of a two-steps reaction that creates two β-ionone rings at each end of the backbone structure (Hirschberg, 2001). Functional prediction of proteins usually involved the identification of sequences or structures similarities between an unknown protein and one or more well-known proteins as well as conserved patterns comparison between the unknown protein sequence with other members with many known sequences and structures (Whisstock and Lesk, 2003). Pfam analysis showed that the *LcyB* sequence isolated from *I. batatas* belongs to the same family as the *LcyE* in the lycopene cyclase protein family. The presence of the Rossmann-fold domain is a distinct feature of this protein family and this domain was found to be present in the *LcyB* sequence (Ashton and Chothia, 2002). The other domain, known as the catalytic domain confers substrate specificity and thus may help in the specificity of the cyclisation process in the pathway.

Signature patterns are usually sites that have important function in the cellular level or contain a distinct pattern only available for a particular protein family. Multiple overlapping signature patterns were observed in the *LcyB* sequence and a higher number

of signature patterns sites were observed compared to the *LcyE* sequence. Similarly, signature pattern sites for casein kinase II phosphorylation, protein kinase C phosphorylation, N-myristoylation and cAMP- and cGMP-dependent protein kinase phosphorylation sites found in *LcyE* sequence were also observed in the *LcyB* sequence. The N-terminal myristoylation was found to play a vital role in membrane targeting and signal transduction in plant responses to environmental stress (Podell and Gribskoy, 2004) and thus may contribute to the difference in carotenoid production level in *I. batatas* compared to other plants.

Previous study on maize endosperm strongly suggested that in the absence of *LcyB* activity, the *LcyE* gene may possessed both the mono and bi-cyclase functions and hence generate both the ε,ψ- and ε,ε-carotenes (Bai *et al*., 2009). This was found to be in agreement with previous studies conducted on *L. sativa, A. aestivalis* and *A. thaliana* in which the *LcyE* gene shown some β-cyclase activity. Overall, high level similarity in the signature patterns were observed between the *LcyE* and *LcyB* sequences. The residual β-cyclase activity possessed by the *LcyE* may be due to the formation of *LcyE* gene from the duplication of an ancient *LcyB* gene (Cunningham and Gantt, 2005; Cunningham *et al*., 2007). Hence, it was predicted that the proportion of β, β-carotenes and β, ε-carotenes produced by the plants can be altered through the different expression of the *LcyE* and *LcyB* genes (Bai *et al*., 2009).

Prot Param analysis predicted a stable 33.4 kDa protein with theoretical pI value of 8.56 and consist mainly leucine (9.8%) and lysine (9.2%). Besides that, *LcyB* amino acid sequence shown 95% homology with the *I.* sp. *Kenyan*, a yellow flower producing plant categorised under the same *Ipomoea* genus. The high homology shared between the *LcyB* sequence with *I.* sp. *kenyan* suggest the highly conserved sequence among

members of the same genus (close relationship). The lower number of amino acid sequence observed for the *LcyB* sequence may suggest differences due to species variation.

Apart from defining the phylogeny relationship between putative genes sequence with sequences from other plants, ClustalW also detect for deletion, insertion or substitution that occur between the sequences. Either naturally occurring or genetically altered by human, these changes have been deeply studied to expand the understanding of the gene functions and the effects of the changes. Several substitutions and deletions were observed in the *LcyB* sequence. A study on the fungi *Phycomyces* genes (*carR*) showed that the introduction of two missence mutations into the gene sequence had caused drastic loss of enzyme activity although these mutations were located far apart in their primary structure. Besides, mutation could also possibly introduce a premature TGA stop codon and resulted in a short and presumably inactive polypeptide, inhibits substrate transfer in the cyclisation process and may increase the susceptibility to chemical inhibition (Arrach *et al*., 2001). Phylogenetic tree showed that the *I. batatas* shared a highly similar *LcyB* sequence with the *I. Kenyan* (located in same cluster) and the high similarity observed between the two sequences may help to predict the possible function of the *LcyB* sequence.

A 956 nucleotides sequence for *Psy* was obtained from the RT-PCR process of the *I. batatas* leaves. The phytoene synthase gene was studied due to its importance in catalysing the conversion of two geranylgeranyl diphosphate (GGPP) molecules to form the phytoene back-bone structure of the carotenoid. The *Psy* sequence in this study showed 91% homology with the *Prunus mume* and *Carica papaya* phytoene synthase sequences and thus confirmed its identity as the putative *Psy* sequence. Compared to the previously isolated *LcyE* and *LcyB* sequences, the *Psy* sequence only showed 88%

homology with the *I.* sp. *Kenyan* which may due plant adaptation to the environment or the differences in the rate of mutation in different genes. This may suggest that the cyclases genes mutate or change in a slower rate compared to the *Psy* gene and thus maintain high homology with plants from the same genus.

Besides, Pfam analysis on *Psy* sequence showed that it belongs to the squalene/phytoene synthase domain and contained two distinct signature patterns; the squalene and phytoene synthases signature 1 and 2 patterns (Hunter *et al*., 2011). Both the squalene synthase EC (SQS) and phytoene synthase EC (PSY) share functional similarities and are mostly reflected at their primary structure level. Three well conserved regions were shared by SQS and PSY which may be involved in either substrate binding and/or catalytic mechanism (Summers *et al*., 1993). The catalytic mechanism is believed to function in the conversion of the GGPP to phytoene in higher plants while substrate binding may contribute to the specificity of the protein. In a study of carotenoid biosynthesis in *Erwinia uredovora*, the addition of a squalene synthase inhibitor (Squalestatin) was observed to cause significant inhibition of phytoene synthase production in the plant (Neudert *et al*., 1998). This could be possibly explained by the possible disruption of the Mn^{2+} and Mg^{2+} ions, which are required for the normal catalytic mechanism of both the squalene and phytoene synthase (Neudert *et al*., 1998). Thus, the specificity in substrate binding is an important characteristic of the *Psy* sequence and plays an important role to organise regulation patterns in the carotenoid biosynthesis pathway. This gene has been in target for genetic manipulation in many plants after the discovery of its functions.

Signature patterns occurred one or more times in isolated sequences and can be used to predict the function and family of a protein. N-glycosylation site and microbodies C-terminal targeting signal were only found in the *Psy* sequence but not in both the

cyclase enzymes. The N-glycosylation site functioned in the addition of N-glycan into plant proteins and lipids and resulted in a great impact on the physiochemical properties and biological function of the plants (Rayon *et al*., 1998). This may function in the interaction between the photosynthetic reaction centre with the plastid of the chloroplast. Microbodies C-terminal on the other hand, helps to direct proteins to peroxisomes, which help in the photorespiration process in leaves (Gould *et al*, 1989). All the signature patterns detected may help either directly or indirectly in the carotenoids biosynthesis in plants.

The theoretical pI values was recorded at 8.6 for the 36.1 kDa long *Psy* protein and thus considered to be a basic protein. Previous study showed that intracellular proteins tend to have negatively charged proteins while nuclear proteins have higher fraction of positively charged residues (Andrade *et al.*, 1998). The pI value recorded came into agreement as the *Psy* was located at the plastid of the chloroplast therefore having a negatively charged protein. The *Psy* sequence however was predicted to be unstable and the instability observed may be due to the absence of post-translational and alternate splicing activity on the final *Psy* protein structure.

No substitution was observed for the squalene and phytoene synthases signature 1 pattern which may define a highly conserved region of the gene sequence. However, a substitution was observed at 174th amino acid residue of the *Psy* sequence and the different amino acid was observed between *I. batatas* with *C. papaya* and *P. mume*. Although previously it was speculated that conserved motifs play important role in maintaining the proper function of a gene, the absence of conserved motif (DEYGNP) in the PgDhn1 sequence that was found consistently in the several known dehydrins sequences may suggest the different roles played by the conserved motif apart from the

common functional activity associated with the gene. Besides, plants may also have regulation systems to complement the loss a conserved motif to facilitate the normal activities.

A study on the lutein content in wheat found that lutein level was associated with the changes of the structural genes and does not involved the changes in the regulators or factors associated with the accumulation process (such as the 'Or' gene in cauliflower) (Lu *et al*., 2006). Hence, substitution at the conserved motif in the *Psy* sequence may provide new information on carotenoid biosynthesis in the *I. batatas*. The *I. batatas* was located at the same cluster as the *I. Kenyan* in the generated phylogenetic tree and hence may indicate high homology in sequences from the same genus. This relationship was found to be similar with the results observed for the *LcyB* sequence.

The study on the molecular aspect of the carotenoids biosynthesis indeed provides new information and understanding in this topic. However, molecular studies to a certain extend are also influenced by external factors succumbed by the plants. Previous studies have documented the influence of environmental factors such as temperature, salt stress and water stress in inducing alternative splicing of transcripts in plants, which directly affects the end product of protein produced (Reddy, 2007; Guo *et al*., 2007). In future, the full-length genes sequences for the *I. batatas* leaves will be determined using the 3' RACE and 5' RACE techniques and the expression level of each gene at the different developmental stages would be conducted via real time PCR technique.

As the conclusion, the lycopene epsilon-cyclase (*LcyE*), lycopene beta-cyclase (*LcyB*) and phytoene synthase (*Psy*) genes were successfully isolated, sequenced and characterised from the *Ipomoea batatas* var. Oren leaves. Sequence homology with other plants in the databases confirmed the identity of the putative sequence. Besides, the relationship between the *I. batatas* sequence and other genes sequences were shown and summarised in the phylogenetic tree.

CHAPTER 7

General Discussion

The usage of *I. batatas* leaves for carotenoid extraction is new and therefore, various questions and challenges have arisen from material selection to the extraction process. This is further coupled with the insufficient information and studies conducted on these leaves in this country. *I. batatas* are planted throughout the year in Malaysia primarily for their storage roots and research conducted on their leaves is scarce. This study is the first ever study conducted to utilise the *I. batatas* leaves in Malaysia.

The identification method via leaf morphologies, which was introduced in this study provide a new approach in identifying the different *I. batatas* varieties. This method was proved to be a good identification tool to differentiate between the varieties and can be complemented with the storage roots' characteristics. Besides, identification of the developmental stages for leaves can also be conducted by referring to the data collected for the width and length of the leaves at the different stages. Since the pigment contents often vary with the developmental stages, this method would be very useful especially in the material selection process.

Among the six Malaysian varieties studied, the *I. batatas* var. Oren leaves were selected as starting material due to the higher levels of lutein and β-carotene pigments in the leaves. These two pigments are important as they contributed mainly to the yellow colour of the carotenoids. However, compared to the other varieties from different parts of the world, the leaves from the Malaysian variety was found to contain lower amount of carotenoid pigments, a trait that may contributed by demographic and environmental factors.

On the other hand, deterioration in leaves sample quality during storage is of major concern due to the large amount of sample collected at a certain time and the short storage duration between leaves collection and extraction process. Storage at lower temperature (15°C) was found to maintain the highest level of pigments in the leaves but temperatures lower than 10°C introduced observable chilling injury to the leaves materials. The leaves can be kept to a maximum of 4 days at 15°C to maintain the quality and pigments contents (approximately 80% remaining pigments) in the leaves before necrosis occur.

Previously, the usage of green leaves as source for carotenoids extraction was not in favour due to the interference of the chlorophyll pigments in the carotenoid extract. Therefore, a suitable method of extraction should remove the chlorophyll entirely from the extract but at the same time does not contribute to carotenoids pigments degradation. Currently, the authorised mixed carotenoids used as yellow dye are obtained from alfalfa through solvent extraction and include the saponification process in the removal of chlorophylls. In this study, the usage of liquid-liquid extraction coupled with a saponification process was observed to be effective in removing the chlorophyll from the leaves extract. This can be indicated by the absence of green colour in the extract. Optimisation study showed that acetone was the most suitable solvent for extraction while 40% potassium hydroxide and 2 hours duration were the best KOH concentration and duration for saponification.

Apart from extraction, the stability of natural dye is also a main concern in natural dye industry. The stability of the carotenoid pigments is crucial because pigments degradation start as soon as the pigments are extracted from the source. Carotenoid degradation and oxidation can occur in the presence of oxygen, light and heat. Greater degradation was observed for pigments kept in high temperature and in the presence of

light. Results from this study showed that the presence of light contributed to a greater degradation of the carotenoids pigments compared to higher temperature. Storage in soybean oil also showed better pigment stability compared to storage in acetone. This indicates that oil-based medium is a potentially good storage medium to maintain the stability of the carotenoid pigments in the extract.

While the study on extraction and stability provide information on the characteristics of the pigments extracted, the study on the composition of this extract is also important to identify the presence of other components in the sample. Qualitative analysis via LCMS/MS conducted on the natural yellow extract showed the presence of carotenoids (lutein, β-carotene, beta cryptoxanthin and 4-ketozeaxanthin), fats (2-monolinoleoylglycerol trimethylsilyl ether and oleoyl) and organic acids (malonic acid, decanoic acid and fumaric acid), which are intermediates and products of normal plant metabolism.

One common problem associated with using leaves as a source is the remaining traces of pesticides after the extraction process. Pesticides are used in the *I. batatas* farming to ensure healthy growth of leaves and storage roots. In this study, herbicides, organophosphorus insecticides and organochlorine insecticides were not detected in the yellow pigments extract and thus confirmed that the extraction protocol used was effective in removing chemicals from the final extracted product. The total removal of these harmful chemicals from the final extract is important in industry to ensure consumers safety.

Common processing methods usually varies in the number of steps and techniques, heating temperature, processing period and storage conditions can significantly affect the phenolic contents, L-ascorbic acid and antioxidant capacity in leaves and fruits.

Antioxidants assay was conducted on both the leaf and yellow extract to determine the effect of the extraction process on the antioxidant capacity of the extract. It was observed that the yellow extract retained most of its antioxidant capabilities after the extraction process, an important characteristic since most antioxidant power is destroyed during production and manufacturing process.

While manipulation of growth conditions and nutritional factors could alter pigments' levels in the leaves to a certain extent, manipulation of the genes involved along the pathway can introduce permanent changes to pigments production in the leaves. The sequence for the lycopene epsilon cyclase (*LcyE*), lycopene beta cyclase (*LcyB*) and phytoene synthase (*Psy*) genes from the carotenoid biosynthesis pathway were successfully characterised from the *I. batatas* leaf. The characterisation of the genes along the pathway will provide new information on the genes and launch a platform for future genetic engineering work on the genes in the hope to increase the production of carotenoids in the leaves with minimal effect on the content in the storage roots.

With regards to economics, the utilisation of the *I. batatas* leaves will generate a total income of approximately $2000 for every harvesting season (for an average 60 hectares plantation area). This will amounted up to approximately $6000 for every small plantation per annum, aside from the sales of the harvested storage roots. This will certainly help to boost the agriculture sector as well as providing consumers with a better natural dye choice.

Generally, all the objectives proposed earlier in this study have been achieved. The optimum extraction conditions and stability of the carotenoids extract was determined and a pesticide free, carotenoids extract with antioxidant properties was successfully extracted from the local *I. batatas* leaves variety. With the information and knowledge

of carotenoid extraction from *I. batatas* leaves, future studies can be conducted to expand this research. Firstly, *I. batatas* varieties from other countries can be incorporated into this study to compare their β-carotene and lutein levels. This can be implemented by collaborating with international research institutes such as the International Potato Centre (ICP). Besides, genetic engineering can also be conducted to control the expression level of the genes involved in the carotenoid biosynthesis. Hence, the production of the desired carotenoid can be controlled and manipulated to fulfil the market demands. For commercialisation purpose, scale-up study can be conducted for industrial application. This will ensure that the quality of the final product from the industry is the same as those performed in lab scale. In addition, since the yellow extract is targeted for the food industry, toxicity studies *in vitro* and *in vivo* will be appropriate to provide sufficient information on its safety level.

As the conclusion, as the global population reaching eight billion and beyond, sustainable agriculture is the way to go in the agriculture industry as well as to protect the environment. From this study, the *I. batatas* leaves, traditionally discarded or used as animal feed after the harvesting season have shown tremendous potential as source of natural yellow pigments. Their low economic value, high antioxidant value and high level of β-carotene and lutein content make the leaves a good and suitable choice for industrial application.

BIBLIOGRAPHY

Abotsi WMK, Woode E, Ainooson GK, Amo-Barimah AK and Boakye-Gyasi E (2010) Antiarthritic and antioxidant effects of the leaf extract of Ficus exasperate P. Beauv (Moraceae). Pharmacogn Res 2(2): 89-97.

Adedapo AA, Jimoh FO, Koduru S, Afolayan AJ and Masika PJ (2008) Antibacterial and antioxidant properties of the methanol extracts of the leaves and stems of *Calpurnia aurea*. BMC Complementary and Alternative Medicine 8: 53.

Agosta W (1996) Bombardier Beetles and Fever Trees: A Close-up Look at Chemical Warfare and Signals in Animals and Plants. Addison-Wesley: Reading, MA.

Alcides RGO, Lucia de Carvalho MJ, Nutti RM, José de Carvalho LV and Fukuda WG (2010) Assessment and degradation study of total carotenoid and ß-carotene in bitter yellow cassava (*Manihot esculenta* Crantz) varieties. Afr J Food Science 4(4): 148-155.

Alfermann AW and Petersen M (1995) Natural product formation by plant cell biotechnology – Results and perspectives. Plant Cell Tiss Org Cult 43: 199–205.

Almeida LB and Penteado MVC (1988) Carotenoids and pro-vitamin A value of white fleshed Brazilian sweet potatoes (*Ipomoea batatas* Lam.). J Food Comp Anal 1: 341–352.

Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215, 403-410.

Ames BM, Shigene MK and Hagen TM (1993) Oxidants, antioxidants and the degenerative diseases of aging. PNAS 90: 7915-7922.

Ampomah-Dwamena C, McGhie T, Wibisono R, Montefiori M, Hellens RP and Allan AC (2009) The kiwifruit lycopene beta-cyclase plays a significant role in carotenoid accumulation in fruit. J Exp Bot 60(13): 3765-3779.

Andersen PC, Brodbeck BV and Mizell III RF (1993) Diurnal variations of amino acids and organic acids in xylem fluid from *Lagerstroemia indica*: an endogenous circadian rhythm. Physiologia Plantarum 89: 783–790.

Andrade MA, O'Donoghue SI and Rost B (1998) Adaptation of protein surfaces to subcellular location. J Mol Biol **276: 517-525.**

Antia BS, Akpan EJ, Okon PA and Umoren IU (2006) Nutritive and Antinutritive Evaluation of Sweet Potato (*Ipomoea batatas*) Leaves. Pak J Nutr 5: 166-168.

Aoki T, Akash T and Ayabe S (2000) Flavonoids of leguminous plants: Structure, biological activity and biosynthesis. J Plant Res 113, 475-488.

Arabnia HR and Tran QN (2011) Software tools and algorithms for biological systems. Advances in Experimental Medicine and Biology. Springer: New York. Pp 59.

Arena E, Fallico B and Maccarone E (2000) Influence of carotenoids and pulps on the color modification of blood orange juice. J of Food Sci 65(3): 458-460.

Arnao MB (2000) Some methodological problems in the determination of antioxidant activity using chromogen radicals: A practical case. Trends Food Sci Tech 11:419–421.

Arnold K, Bordoli L, Kopp J and Schwede T (2006) The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling. Bioinformatics 22: 195-201.

Arrach N, Fernández-Martín R, Cerdá-Olmedo E and Avalos J (2001) A single gene for lycopene cyclase, phytoene synthase, and regulation of carotene biosynthesis in Phycomyces. PNAS 98(4): 1687- 1692.

Ascherio A, Rimm EB, Giovannucci EL, Colditz GA, Rosner B, Willett WC *et al.* (1992) A prospective study of nutritional factors and hypertension among US men. Circulation 86: 1475-1484.

Ashton M and Chothia C (2002) The geometry of domain combination in proteins. J Mol Bio 315: 927-939.

Attwood TK and Parry-Smith DJ (1999) Introduction to Bioinformatics. Addison Wesley Longman.

Bae SH and Suh HJ (2007) Antioxidant activities of five different mulberry cultivars in Korea. LWT-Food Sci Tech 40: 955-962.

Bai L, Kim EH, DellaPenna D and Brutnell TP (2009) Novel lycopene epsilon cyclase activities in maize revealed through perturbation of carotenoid biosynthesis. Plant J 59, 588–599.

Bailey WE (2011) Sweet Potatoes. Retrieved on 16 May 2011, from http://www. sweetpotatoes.com /About /VarietiesandBotanicalInformation.aspx

Barros L, Baptista P and Ferreira ICFR (2007) Effect of *Lactarius piperatus* fruiting body maturityl stage on antioxidant activity measured by several biochemical assays. Food Chem Toxicol 45: 1731-1737.

Barry P, Evershed RP, Young A, Prescott MC and Britton G (1992) Characterization of carotenoid acyl ester produced in drought-stressed barley seedlings. Phytochem 31: 3163–3168.

Bartley G and Scolnik P (1995) Plant carotenoids: pigments for photoprotection, visual attraction, and human health. Plant Cell 7: 1027-1038.

Barzana E, Rubio D, Santamaria RI, Garcia-Correa O, Garcia F, Ridaura Sanz VE *et al.* (2002) Enzyme-mediated solvent extraction of carotenoids from marigold flower (*Tagetes erecta*). J Agric Food Chem 50: 4491-4496.

Bateman A, Birney E, Cerruti L, Durbin R, Etwiller L, Eddy SR *et al.* (2002) The Pfam protein families database. Nucleic Acids Res 30(1), 276-80.

Bauernfeind JC (1972) Carotenoid vitamin A precursors and analogs in foods and feeds. J Agric Food Chem 20: 456-473.

Bauernfeind JC, Brubacher GB, Klaui HM and Marusich WL (1971) Use of carotenoids, In: Isler O, Gutmann H and Solms U (eds.). Carotenoids. Birkhauser: Basel. Chapter 11.

Beecher GR and Khachik F (1984) Evaluation of vitamin A and carotenoid data in food composition tables. J Natural Cancer Institute 73: 1397-1404.

Ben-Amotz A and Avron M (1990) The biotechnology of cultivating the halotolerant alga *Dunaleilla*. Trends Biotechnol 8: 121-126.

Ben-Amotz A and Fishler R (1998) Analysis of carotenoids with emphasis on 9-cis β-carotene in vegetables and fruits commonly consumed in Israel. Food Chem 62(4): 515-520.

Bendich A (1993) Biological functions of dietary carotenoids, In: Canfield LM, Krinsky NI and Olson JA (ed.). Carotenoids in Human Health. Ann NY Acad Sci: New York. Vol 691, pp. 61-67.

Berlin J (1986) Secondary products from plant cell cultures, In: Rehm HJ and Reed G (eds). Biotechnology a comprehensive treatise. Verlag Chemie, Verlagsgesellschaft: Weinheim. Vol 4, pp. 630–658.

Bhaskarachary K, Sankar Rao DS, Deosthales YG and Reddy V (1995) Carotene content of some common and less familiar foods of plant origin. Food Chem 54: 189-193.

Bhattacharya M, Fuhrman L and Ingram A (1995) Single-run separation and detection multiple metabolic intermediates by anion-exchange high-performance liquid chromatography and application to cell pool extracts prepared from *Escherichia coli*. Anal Biochem 232: 98–106.

Biacs AP, Czinkotai B and Hoscheke A (1992) Factors affecting stability of coloured substances in paprika powders. J Agric Food Chem 40(3): 363-367.

Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG and Gluud C (2007) Mortality in Randomized Trials of Antioxidant Supplements for Primary and Secondary Prevention. Systematic Analysis and Meta-Analysis 297(8): 842-857.

Bjorkman O (1973) Comparative studies of photosynthesis in higher plants. Photophys 8: 1-63.

Blois MS (1958) Antioxidant determinations by the use of a stable free radical. Nature 181(1): 199-200.

Bone RA and Landrum JT (1992) Distribution of macular pigment components, zeaxanthin and lutein, In: Pasker L (ed.). Methods in Enzymology. Academic Press: San Diego. Pp 360-366.

Borchetia S, Bora C, Gohain B, Bhagawati P, Agarwala N, Bhattacharya N *et al.* (2011) Cloning and Heterologous Expression of a Gene encoding Lycopene-epsilon-cyclase, a precursor of lutein in tea (*Camellia sinensis* var assamica). Afr J Biotechnol 10(32): 5934-5939.

Bourgaud F, Gravot A, Milesi S and Gontier E (2001) Production of plant secondary metabolites. Plant Sci 161: 839-851.

Bouvier F, Keller Y, d'Harlingue A and Camara B (1998) Xanthophyll biosynthesis: molecular and functional characterization of carotenoid hydroxylases from pepper fruits (*Capsicum annuum* L). Biochim Biophys Acta 1391: 320-328.

Bowen PE (2005) Selection of surrogate endpoint biomarkers to evaluate the efficacy of lycopene/tomatoes for the prevention/progression of prostate cancer. J Nutr 135: 2068S-2070S.

Bowler C, Neuhaus G, Yamagata H and Chua NH (1994) **Cyclic GMP and calcium mediate phytochrome phototransduction. Cell 77(1): 73-81.**

Bramley P, Teulieres C, Blain I, Bird C and Schuch W (1992) Biochemical characterization of transgenic tomato plants in which carotenoid synthesis has been inhibited through the expression of antisense RNA to pTOM5. Plant J 2: 343-349.

Bravo L (1998) Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev 56: 317-333.

Britton G (1983) The biochemistry of natural pigments. Cambridge University Press: Great Britain. Pp 3-22.

Britton G (1995) Structure and properties of carotenoids in relation to function. FASEB Journal 9: 1551-1558.

Britton G, Liaen-Jensen S and Pfander H (1995) The carotenoids. In Biel-Benken (Volume 1A), Isolation and analysis. Birkhauser-Verlag: Biel-Benken, Switzerland. Pp 199-214.

Buitelaar RM and Tramper J (1992) Strategies to improve the production of secondary metabolites with plant cell cultures – A literature review. J Biotechnol 23: 111–141.

Bulda OV, Rassadina, VV, Alekseichuk HN and Laman NA (2008) Spectrophotometric measurement of carotenes, xanthophylls, and chlorophylls in extracts from plant seeds. Russian J Plant Phy 55(4): 544-551.

Butt E, Abel K, Krieger M, Palm D, Hoppe V, Hoppe J *et al.* (1993) cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets. J Bio Chem 269(20): 14509-14517.

Campo GM, Avenoso A, Campo S, Ferlazzo AM, Altavilla D and Calatroni A (2003) Efficacy of treatment with glycosaminoglycans on experimental collagen-induced arthritis in rats. Arthritis Res Ther 5: 122-131.

Cao G, Sofic E and Prior RL (1997) Antioxidant and prooxidant behavior of flavonoids: Structure-activity relationships. Free Radicals Biol Med 22: 749-760.

Ceron-Garcia MC, Campos-Perez I, Macias-Sanchez MD, Bermejo-Roman R, Fernandez-Sevilla JM and Molina-Grima E (2010) Stability of carotenoids in *Scenedesmus almeriensis* biomass and extracts under various storage conditions. J Agric Food Chem 58: 6944–6950.

Chamovitz D, Pecker I, Sandmann G, Boeger P and Hirschberg J (1990) Cloning a gene for norflurazon resistance in cyanobacteria. Z Naturforsch 45c: 482-486.

Chan A, Puiu D, Melake A, Orvis J, Zhao Q, Wortman J *et al*. (2009) Direct submission to Genbank. Available at http://www.ncbi.nlm.nih.gov/protein/XP_00 25141 36.1

Chandler LA and Schwartz SJ (1987) HPLC separation of cis –transcarotene isomers in fresh and processed fruits and vegetables. J Food Sci 52: 289–294.

Chen BH and Chen YY (1992) Determination of carotenoids and chlorophylls in water convolvulus (*Ipomoea aquatica*) by liquid chromatography. Food Chem 45: 129-134.

Chen H, Zuo Y and Deng Y (2001) Separation and determination of flavonoids and other phenolic compounds in cranberry juice by high performance liquid chromatography. J Chromatogr A 913: 387-395.

Chen HB and Chen YY (1993) Determination of carotenoids in sweet potato leaves during microwave cooking. J Agric Food Chem 41(8): 1315-1320.

Chen HE, Peng HY and Chen BH (1995) Stability of carotenoids and vitamin A during storage of carrot juice. Food Chem 57(4): 497-503.

Chen JP, Tai CY and Chen BH (2004) Improved liquid chromatographic method for determination of carotenoids in Taiwanese mango (*Mangifera indica* L.). J Chromatogr A 1054: 261-268.

Chrzanowski G, Sempruch C and Sprawka I (2007) Investigation of phenolic acids in leaves of blackcurrant (*Ribes nigrum* L.) and sour cherry (*Prunus cerasus* L.). J Polish Agric Uni 10(4).

Cinar I (2004) Carotenoid pigment loss of freeze-dried plant samples under different storage conditions. Lebensm-Wiss. u.-Technol. 37: 363-367.

Consultative Group on International Agricultural research (CGIAR) (2005) Research & Impact: Sweet Potato. Retrieved on 15 June 2010, from http://www.cgiar. org/impact /research/sweetpotato.html

Consumer affairs (2010) Consumer groups presses for ban on food dye. Retrieved on 8 June 2010, from http://www.consumeraffairs.com/news04/2008/06/cspi_food _dye. html.

Craft NE and Granado F (1993) Effect of sample preparation on fat-soluble vitamins and carotenoid concentrations. FASEB J 7: A520.

Croteau R, Kutchan TM and Lewis NG (2000) Natural products (Secondary Metabolites), In: Buchanan B, Gruissem W and Jones R (eds.). Biochemistry & Molecular Biology of Plants. American Society of Plant Physiologists. Pp 1250-1318.

Cullis CA and Creissen GP (1987) Genome organization and variation in higher plants. Annals Bot 60(Supp. 4): 103-113.

Culp SJ and Beland FA (1996) Malachite green: a toxicological review. J American College of Tech 15: 219–238.

Cunningham FX Jr and Gantt E (2001) One ring or two? Determination of ring number in carotenoids by lycopene epsilon-cyclases. PNAS 98: 2905–2910.

Cunningham FX Jr and Gantt E (2005) A study in scarlet: enzymes of ketocarotenoid biosynthesis in the flowers of *Adonis aestivalis*. Plant J 41: 478–492.

Cunningham FX Jr, Lee H and Gantt E (2007) Carotenoid biosynthesis in the primitive red alga *Cyanidioschyzon merolae*. Eukaryot Cell 6: 533–545.

Cutler R (1984) Carotenoids and retinol: their possible importance in determining longevity of private species. PNAS 81: 7627-7631.

Davison PA, Hunter CN and Horton P (2002) Overexpression of beta-carotene hydroxylase enhances stress tolerance in *Arabidopsis*. Nature 418: 203–206.

de Castro E, Sigrist CJ, Gattiker A, Bulliard V, Langendijk-Genevaux PS, Gasteiger E *et al.* (2006) ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. Nucleic Acids Res 34: W362-5.

Del Campo JA, García-González M, Guerrero MG (2007) Outdoor cultivation of microalgae for carotenoid production: current state and perspectives. Appl Microbiol Biotechnol 74: 1163–1174.

Del Campo JA, Moreno J, Rodríguez H, Vargas MA, Rivas J and Guerrero MG (2000) Carotenoid content of chlorophycean microalgae: factors determining lutein accumulation in *Muriellopsis sp*. (Chlorophyta). J Biotechnol 76: 51–59.

Del Campo JA, Rodriguez H, Moreno J, Vargas MA, Rivas J and Guerrero MG (2001) Lutein production by *Muriellopsis sp*. in an outdoor tubular photobioreactor. J Biotechnol 85: 289–295.

Demmig-Adams B, Gilmore AM and Adams WW (1996) Carotenoids 3: in vivo function of carotenoids in higher plants. FASEB J 10: 403–412.

Demming-Adams B and Adams WW III (2002) Antioxidants in photosynthesis nutrition. Science 298: 2149–2153.

Dickinson TA, Parker WH and Strauss RE (1987) Another approach to leaf shape comparisons. Taxonomy 36(1): 1-20.

Dixon RA (1999) Isoflavonoids: biochemistry, molecular biology and biological functions, In: Barton DHR, Nakanishi K and Meth-Cohn O (eds.-in chief). Comprehensive natural products chemistry, Vol. 1- Polyketides and Other Secondary Metabolites Including Fatty Acids and Their Derivatives. Elsevier: Amsterdam. Pp 773–824.

Dixon RA (2001) Natural products and plant disease resistance. Nature 411: 843–847.

Dreosti IE (2000) Antioxidant polyphenols in tea, cocoa and wine. Nutrition 16: 692–694.

Droge W (2002) Free radicals in the physiological control of cell function. Review Physiol Rev 82: 47-95.

Ducreux LJM, Morris WL, Hedley PE, Shepherd T, Davies HV, Millam S *et al.* (2005) Metabolic engineering of high carotenoid potato tubers containing enhanced levels of beta-carotene and lutein. J Exp Bot 56: 81–89.

Dudareva N, Pichersky E and Gershenzon J (2004) Biochemistry of plant volatiles. Plant Physiol 135: 1893–1902.

Dweyer JH, Navab M, Dwyer KM, Hassan K, Sun P, Shircore A *et al*. (2001) Oxygenated carotenoid lutein and the progression of early atherosclerosis. The Los Angeles atherosclerosis study. Circulation 103: 2922–2927.

Edge R, McGarvey DJ and Truscott TG (1997) The carotenoids as anti-oxidants–a review. **J Photochem Photobiol B Biol** 41: 189–200.

Edwards AJ, Nguyen CH, You CS, Swanson JE, Emenhiser C and Parker RS (2002) α- and β-Carotene from a commercial carrot puree are more bioavailable to humans than from boiled-mashed carrots, as determined using an extrinsic stable isotope reference method. J Nutr 132(2): 159-167.

Eggink LL, Park HS and Hoober JK (2001) The role of chlorophyll *b* in photosynthesis: Hypothesis. BMC Plant Biology 1(2)

Engelmann NJ, Clinton SK and Erdman Jr JW (2011) Nutritional Aspects of Phytoene and Phytofluene, Carotenoid Precursors to Lycopene. Adv Nutr 2(1): 51–61.

FAOSTAT (Food and Agricultural Organisation of the United Nations) (2012) Retrieved on 28 March 2012, from http://faostat.fao.org/

Ferruzzi MG, Sander LC, Rock CL and Schwartz SJ (1998) Carotenoid determination in biological microsamples using liquid chromatography with a coulometric electrochemical array detecor. Anal Biochem 256: 74–81.

Fessenden RJ and Fessenden JS (1990) Organic Chemistry (4th ed.). Brooks/Cole: California.

Fraser PD, Kiano JW, Truesdale MR, Schuch W and Bramley PM (1999) Phytoene synthase-2 enzyme activity in tomato does not contribute to carotenoid synthesis in ripening fruit. Plant Mol Bio 40: 687-698.

Fraser PD, Truesdale MR, Bird CR, Schuch W and Bramley PM (1994) Carotenoid biosynthesis during tomato fruit development. Plant Physiol 105, 405–413.

Frenich AG, Torres MEH, Vega AB, Vidal JLM and Bolaños PP (2005) Determination of ascorbic acid and carotenoids in food commodities by liquid chromatography with mass spectrometry detection. J Agric Food Chem 53: 7371-7376.

Fullas F (2001) Ethiopian traditional medicine: Common medicinal plants in perspective (1st ed.). United States of America.

Gabas AL, Telis-Romero J and Menegalli FC (2003) Cinética de degradação do ácido ascórbico em ameixas liofilizadas. Ciência Tecnologia de Alimentos 23: 66-70.

Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD and Bairoch A (2003) ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res 31: 3784-3788.

Giovannucci E (1999) Tomatoes, tomato-based products, lycopene, and cancer: review of the epidemiologic literature. J National Cancer Institute 91: 317-331.

Giuliano1 G, Tavazza1 R, Diretto G, Beyer P and Taylor MA (2008) Metabolic engineering of carotenoid biosynthesis in plants. Review Cel-Press (CHECK) 139-145.

Goodwin TW (1980) The Biochemistry of the Carotenoids, (2nd ed). Chapman and Hall: London. Vol. 1

Gordon MH and An J (1995) Antioxidant activity of flavonoids isolated from licorice. J Agric Food Chem 43: 1784-1788.

Gould SJ, Keller G, Hosken N, Wilkinsin J and Subramani S (1989) A conserved Tripeptide Sorts Proteins to Peroxisomes. J Cell Bio 108: 1657-1664.

Granado F, Olmedilla B and Blanco I (2003) Nutritional and clinical relevance of lutein in human health. British J Nutr 90: 487-502.

Granado F, Olmedilla B, Gil-Martinez E and Blanco I (2001) A fast, reliable and low cost saponification protocol for analysis of carotenoids in vegetables. J Comp Anal 14: 479-489.

Grünewald K, Hirschberg J and Hagen C (2001) Ketocarotenoid biosynthesis outside of plastids in the unicellular green alga *Haematococcus pluvialis*. J Biol Chem 276: 6023-6029.

Guaratini T, Vessecchi R, Pinto E, Colepicolo P and Lopez NP (2005) Balance of xanthophylls molecular and protonated molecular ions in electrospray ionization. J Mass Spectrom 40: 963-968.

Guo XQ, Zhang HZ and Li DB (2007) Alternative splicing of pre-mRNA in plants. Chin J Agri Biotechnol 4: 1–8.

Guzman I, Hambly S, Romero J, Bosland P and O'Connell M (2010) Variability of carotenoid biosynthesis in orange coloured *Capsicum* spp. Plant Sci 179: 49-59.

Hajimahmoodi M, Sadeghi N, Jannat B, Oveisi MR, Madani S and Kiayi M (2008) Antioxidant cctivity, reducing power and total phenolic content of Iranian olive cultivar. J Bio Sci 8(4): 779-783.

Hall A, Bockett G and Nahdy S (1998) Sweetpotato postharvest systems in Uganda: Strategies, constraints and potentials, In: International Potato Center (CIP) Social Science Department Working Paper No. 1998-7. CIP, Lima, Peru.

Harborne JB and Baxter H (1993) Phytochemical Dictionary, A Handbook of Bioactive Compounds from Plants. Taylor & Francis: London.

Harborne JB and Williams CA (2000) Advances in flavonoid research since 1992. Phytochem 55: 481-504.

Hardwin C (2010) Tartrazine- 30 years of Poisoning by the most common food additives. Retrieved on 21 March 2010, from http://www.natural-health-for-you.com /tartrazine.html.

Hartemink AE, Poloma S, Maino M, Powell KS, Egenae J and Sullivan JN (2000) Yield decline of sweet potato in the humid lowlands of Papua New Guinea. Agric Ecosyst Environ 79(3): 259-269.

Henry LK, Catignani GL and Schwartz SJ (1998) Oxidative degradation kinetics of lycopene, lutein and 9-*cis* and all-*trans* β-Carotene. J Am Oil Chem Soc 75: 823-829.

Hertog MGL, Feskens EJM, Hollman PCH, Katan MB and Kromhoutd D (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. Lancet 342: 1007-1011.

Hirchberg J (2001) Carotenoid biosynthesis in flowering plants. Curr Opin Plant Bio 4: 210-218.

Hirschberg J (1998) Molecular biology of carotenoid biosynthesis, In: Britton G, Liaaen-Jensen S and Pfander H (eds.). Carotenoids Vol 3. Birkhauser Verlag: Basel. Pp 149-194.

Holm L and Sander C (1997). An evolutionary treasure: Unification of a broad set of Amidohydrolases related to urease. PROTEINS: Structure, Function, and Genetics 28: 72–82.

Hostettmann K, Wolfender JL and Terreaux C (2001) Modern screening techniques for plant extracts. Pharm Bio 39: 18-32.

Hotta H, Nagano S, Ueda M, Tsujino Y, Koyama J and Osakai T (2002) Higher radical scavenging activities of polyphenolic antioxidants can ascribed to chemical reactions following their oxidation. Biochem Biophys Acta 1572: 123-132.

Huck CW, Popp M, Scherz H, Bonn GK (2000) Development and evaluation of a new method for the determination of the carotenoid content in selected vegetables by HPLC and HPLCMS-MS. J Chrom Sci 38: 441–449.

Hunter S, Jones P, Mitchell A, Apweiler R, Attwood TK, Bateman A *et al*. (2011) Interpro in 2011: new developments in the family and domain prediction database. Nucleic Acid Research 40(1): D306-D312.

International Potato Center (ICP) (2006) Sweetpotato. Retrieved on 3 May 2010, from http://www.cipotato.org/sweetpotato/

Ishida H, Suzuno H, Sugiyama N, Innami S, Tadokoro T and Maekawa A (2000) Nutritive evaluation on chemical components of leaves, stalks and stems of sweet potatoes (*Ipomoea batatas poir*). Food Chem 68: 359-367.

Ishiguro K and Yoshimoto M (2007) Lutein content of sweetpotato leaves II. Cultivar differences, distribution in leaves at different positions and changes during storage. National Agricultural Research Centre for Kyushu Okinawa region (KONARC). Sweetpotato Research Front 20: 4.

Islam MS, Yoshimoto M, Yahara S, Okuno S, Ishiguro K and Yamakawa O (2002) Identification and characterization of foliar polyphenolic composition in sweetpotato (*Ipomoea batatas* L.) genotypes. J Agric Food Chem 50(13): 3718-3722.

Islam S (2006) Medicinal and nutritional qualities of sweetpotato tops and leaves. Plant Sci FSA135.

Jayawickrama DA and Sweedler JV (2003) Hyphenation of capillary separations with nuclear magnetic resonance spectroscopy. J Chromatogr A 1000*:* 819-840.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) (2006) WHO/FAO Joint Expert Committee on Food Additives. Safety evaluation of certain food additives. WHO Food Additives Series 54: 49-86. Available at: http://www.inchem .org/documents/jecfa /jecmono/v54je01.pdf

Jewell VC, Sweet D, Tubman TRJ, Northrop-Clewes CA and Thurnham DI (2000) Lutein and zeaxanthin levels in newborn infants and their mothers. Proc Nutr Soc 59: 47A.

Jia MY, Kim HJ and Min DB (2007) Effects of soybean oil and oxidized soybean oil on the stability of β-carotene. Food Chem 103: 695-700.

Jia ZS, Tang MC and Wu JM (1999) The determination of flavonoids contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 64: 555-559.

Jiang TS and Pearce D (2005) Shelf-life extensions of leafy vegetables: evaluating the impacts, ACIAR Project PHT/1994/016. Australian Centre for International Agricultural Research. Retrieved on 2 Sep 2011, from www.aciar.gov.au.

John B (2008) Synthetic dye: A look at Environmental & Human Risks. Retrieved on 14 March 2010, from http://greencotton.wordpress.com/

Johnson MP, Davison PA, Ruban AV and Horton P (2008) The xanthophyll cycle pool size controls the kinetics of non-photochemical quenching in *Arabidopsis thaliana.* FEBS Letters 582: 262–266.

Johnson-Down L, Saudny-Unterberger H and Gray-Donald K (2002) Food habits of Canadians: Lutein and lycopene intake in the Canadian population. J Am Diet Assoc 102: 988-991.

Jones DL (1998) Organic acids in the rhizosphere-a critical review. Plant Soil 205: 25–44.

Jothi D (2008) Extraction of natural dyes from African Marigold flower (*Tagetes erecta* L.) for textile colouration. AUTEX Res J 8(2): 49-53.

Kakaty BM, Thamburaj S and Stalin P (1992) Study on the gowth and yield of sweet potato at different stages of harvest. J Root Crops 18: 73-76.

Karaivanova M, Drenska D and Ovcharov R (1990) A modification of the toxic effects of platinum complexes with anthocyanins. Eksperimetnalna Meditsna I Morfologiia 29: 19–24.

Karatas AD, Aygun D and Baydin A (2006) Characteristics of endosulfan poisoning: a study of 23 cases. Singapore Med Journal 47(12): 1030-1032.

Karawita R, Siriwardhana N, Lee KW, Heo MS, Yeo IK, Lee YD *et al*. (2005) Reactive oxygen species scavenging, metal chelation, reducing power and lipid peroxidation inhibition properties of different solvent fractions from *Hizikia fusiformis*. Eur Food Res Technol 220: 363-371.

Khachatryan A, Bansode RR, Labonte DR and Losso JN (2003) Identification of sweet potato leaves (*Ipomoea batatas*) as an excellent source of lutein. Nutraceutical and Functional Food Conference 88: 7.

Khachik F, Goli MB, Beecher GR, Holden J, Lusby WR, Tenorio MD *et al*. (1992) Effect of food preparation on qualitative and quantitative distribution of major carotenoid constituents of tomatoes and several green vegetables. J Agric Food Chem 40: 390-398.

Kidmose U, Yang RY, Thilsted SH, Christensen LP and Brandt K (2006) Content of carotenoids in commonly consumed Asian vegetables and stability and extrability during frying. J Food Compos Ana. 19: 562–571.

Kim YN, Giraud DW and Driskell JA (2007) Tocopherol and carotenoid contents of selected Korean fruits and vegetables. J Food Compos Anal 20: 458–465.

Kimura M and Rodriguez-Amaya DB (2002) A scheme for obtaining standards and HPLC quantification of leafy vegetable carotenoids. J Food Chem 78: 389-398.

Kimura M, Kobori CN, Rodriguez-Amaya DB and Penelope N (2005) Screening and HPLC methods for carotenoids in Sweet Potato, Cassava, and maize for plant breeding trials. J Food Chem 100: 1734-1746.

Kimura M, Rodriguez-Amaya DB and Godoy HT (1990) Assessment of the saponifcation step in the quantitative determination of carotenoids and provitamins A. Food Chem 35: 187-195.

Kishimoto A, Nishiyama K, Nakanishi H, Uratsuji Y, Nomura H, Takeyama Y *et al*. (1985) Studies on the phosphorylation of myelin base protein by Protein Kinase C and Adenosine 3:5-Monophosphate-dependent Protein Kinase. J Bio Chem 250(23): 12492-12499.

Kita M, Kato M, Ban Y, Honda C, Yaegaki H, Ikoma Y *et al.* (2007) Carotenoid accumulation in Japanese apricot (*Prunus mume* Siebold & Zucc.): Molecular analysis of carotenogenic gene expression and ethylene regulation. J Agric Food Chem 55(9): 3414-3420.

Klimezka J and Irzyniec Z (1997) Effect of temperature on the rate of vitamin C decomposition in blanched Brussels sprouts during frozen storage. Cholnictwo 32: 37–40.

Köllner TG, Schnee C, Gershenzon J and Degenhardt J (2004) The variability of sesquiterpenes emitted from two *Zea mays* cultivars is controlled by allelic variation of two terpene synthase genes encoding stereoselective multiple product enzymes. Plant Cell 16: 1115–1131.

Konczak-Islam I, Yoshimoto Y, Hou D, Terahara N and Yamakawa O (2003) Potential chemopreventive properties of anthocyanin-rich aqueous extracts from in vitro produced tissue of sweetpotato. J Agric Food Chem 51: 5916–5922.

Koo HM and Mohamed S (2001) Flavonoid (Myricetin, Quercetin, Kaempferol, Luteolin, and Apigenin) content of edible tropical plants. J Agric Food Chem 49: 3106-3112.

Kopas-Lane LM and Warthesen JJ (1995) Carotenoid photostability in raw spinach and carrots during cold storage. J Food Sci 60: 773–776.

Korber B and Myers G (1992) Signature pattern analysis: a method for assessing viral sequence relatedness. AIDS Res Hum Retroviruses. **8(9): 1549-60.**

Krinsky NI and Yeum KJ (2003) Carotenoid-radical interactions. Biochem Biophys Res Commun 305(3): 754-760.

Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkosi AE and Hilpert KF (2002) Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. Am J Med 113: 71S-88S.

Kumar NVR, Joy KL, Kuttan G, Ramsewak RS, Nair MG and Kuttan R (2002) Antitumour and anticarcinogenic activity of Phyllanthus amarus extract. J Ethnophar 81: 17–22.

Küpper H, Seibert S and Parameswaran A (2007) Fast, sensitive, and inexpensive alternative to analytical pigment HPLC: quantification of chlorophylls and carotenoids in crude extracts by fitting with Gauss peak spectra. Anal Chem. 79(20): 7611-7627.

Kyte J and Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Bio 157: 105-132.

Landrum JT and Bone RA (2001) Lutein, zeaxanthin, and the macular pigment. Arc Biochem Biophy 385: 28-40.

Lange DD and Cameron AC (1994) Postharvest shelf life on sweet basil (*Ocimum basilicum*). HortSci 29: 102–103.

Lansdown R and Yule W (1984) The Lead debate: the environment, toxicology and child health. Mackays of Chatham Ltd: Great Britain.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H *et al*. (2007) ClustalW and ClustalX version 2. Bioinformatics 23(21): 2947-2948.

Larson RA (1988) The antioxidants of higher plants. Phytochem 4: 969-978.

Lasellier HE, Marty C and Lebert A (1993) Analysis of carotenoids by high-performance liquid chromatography and supercritical fluid chromatography. J Chromat 633: 9-23.

Latasa M and Bidigare RR (1998) A comparisons of phytoplankton populations of the Arabian Seas during the Spring Intermonsoon and Southwest monsoon of 1995 as described by HPLC-analysed pigments. Deep-Sea Res II, 45: 2133-2170.

Leffingwell JC (2002) Tobacco. Leffingwell Reports 2(6), October 2002 issue. (Online publication) from, http://www.leffingwell.com/download/tobacco.pdf.

Lefsrud M, Kopsell D, Wenzel A and Sheehan J (2007) Changes in kale (*Brassica oleracea L*. var. acephala) carotenoid and chlorophyll pigment concentrations during leaf ontogeny. Sci Hort 112: 136-141.

Liao M and Chen X (2006) Cloning of genes for partial member enzymes of carotene biosynthetic pathway in papaya (*Carica papaya* L.) (Unpublished) Direct Submission to Genbank. Available at: http://www.ncbi.nlm.nih.gov/protein/11034 8215? report=genbank&log$=prottop&blast_rank=12&RID=C1JX6MJ3016

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol 148: 349–382.

Lichtenthaler HK and Buschmann C (2001) Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy. Current Protocols in Food Analytical Chemistry F4.3.1 – F4.3.8.

Lichtenthaler HK, Rohmer M and Schwender J (1997) Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. Physio Plantarum 101: 643-652.

Lietti A, Cristoni A and Picci M (1976) Studies of *Vaccinium myrtillus* anthocyanosides. I. Vasoprotective and anti-inflammatory activity. Arzneimittel-Forschung 26(5): 829–832.

Lin CH and Chen BH (2005) Stability of carotenoids in tomato juice during storage. Food Chem 90: 837-846.

Liu CB, Tian L, Yu ZX, Zhang FC and Chen X (2009) Effects of solvents on extraction of effective components from cell wall tissues of Chinese traditional medicines. Chem Res Chinese Universities 25(6): 899-903.

Liu YT, Perera CO and Suresh V (2007) Comparison of three chosen vegetables with others from South East Asia with others for their lutein and zeaxanthin content. Food Chem 101(4): 1533-1539.

Longe OG (1986) Energy Content of some tropical starch crop in Nigeria. J Agric 21: 134-136.

Longnecker MP, Rogan WJ and Lucier G (1997) The human health effects of DDT (Dichlorodiphenyl trichloroethane) and PCBS (Polychlorinated biphenyls) and an overview of organochlorines in public health. Annu Rev Publ Health 18: 211-244.

Lopez-Bucio J, Nieto-Jacobo MF, Ramirez-Rodriguez V and Herrera-Estrella L (2000) Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. Plant Sci 160: 1-13.

Lopez-Hernandez E, Ponce-Alquicira E, Cruz-Sosa F and Guerrero-Legarreta I (2001) Characterization and stability of pigments extracted from *Terminalia Catappa* leaves. J Food Sci 66(6): 832-836.

Lotan T and Hirschberg J (1995) Cloning and expression in *Escherichia coli* of the gene encoding b-C-4-oxygenase that converts β-carotene to the ketocarotenoid canthaxanthin in *Haematococcus pluvialis*. FEBS Lett 364: 125-128.

Lu S, Van Eck J, Zhou X, Lopez AB, O'Halloran DM, Cosman KM, *et al* (2006) The cauliflower or gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of {beta}-carotene accumulation. Plant Cell 18: 3594–3605.

Ludvik BH, Mahdjoobian K, Waldhaeusl W, Hofer A, Prager R, Kautzky-willer A et al. (2000) The effect of *Ipomoea batatas* (Caiapo) on glucose metabolism and serum cholesterol in patients with Type 2 Diabetes. Diabetes care 25(1): Jan 2002.

Ma M, Hong CL, An SQ and Li B (2003) Seasonal, spatial, and interspecific variation in quercetin in Apocynum venetum and Poacynum hendersonii, Chinese traditional herbal teas. J Agric Food Chem 51: 2390-2393.

Mangels AR, Holden JM, Beecher GR, Forman MR and Lanza E (1993) Carotenoid content of fruits and vegetables: an evaluation of analytic data. J Am Diet Assoc 93: 284-296.

Marin E, Nussaume L, Quesada A, Gonneau M, Sotta B, Hugueney P, *et al* (1996) Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*. EMBO J 15: 2331-2342.

Marinova D, Ribarova F and Atanassova M (2005) Total phenolics and total flavonoids in Bulgarian fruits and vegetables. J Uni Chem Tech Metallurgy 40(3): 255-260.

Marz U (2005) The Global Market for Carotenoids. Business Communication, Inc.

McCollum JP (1954) Effects of light on the formation of carotenoids in tomato fruits. Food Res 19: 182-189.

McEwen MJ (2004) The sweet potato (*Ipomoea batatas*) from early migration to future conservation (The Worlds Most Under-Rated Crop), BSc in Habitat and Soil Management, University of Reading (2000)

Meijden EVR (1996) Plant defence, an Evolutionary Dilemma: contracting effects of (specialist and generalist) herbivores and natural enemies. Entomologia Experimentalis et Applicata 80: 307-310.

Menelaou E, Kachatryan A, Losso JN, Cavalier M and Bonte DL (2006) Lutein content in sweetpotato leaves. Hort Sci 41(4): 1269-1271.

Mercadante AZ and Rodriguez-Amaya DB (1991) Carotenoid Composition of a Leafy Vegetable in Relation to Some Agricultural Variables. J Agric Food Chem 39: 1094-1097.

Minguez-Mosquera MI and Gandul-Rojas B (1994) Mechanism and kinetics of carotenoid degradation during the processing of green table olives. J Agric Food Chem 42: 1551–1554.

Minguez-Mosquera MI and Jaren-Galan M (1995) Kinetics of the Decolouring of Carotenoid Pigments. J Sci Food Agric 67: 153–161.

Mínguez-Mosquera MI and Gandul-Rojas B (1995) High-performance liquid chromatographic study of alkaline treatment of chlorophyll. J Chro A 690(2): 161-176.

Misawa N and Shimada H (1998) Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts. J Biotechn 59: 169–181.

Moehs CP, Tian L, Osteryoung KW and DellaPenna D (2001) Analysis of carotenoids biosynthetic gene expression during marigold petal development. Plant Mol Biol 45: 281-293.

Moreau RA, Whitaker BD and Hicks KB (2002) Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. Prog Lipid Res **41**: 457-500.

Moreno-Alvarez MJ, Belén CDR and Torrez V (2000). Degradación cinética de carotenoides obtenidos de frutos de Carica papaya L. Revista da Faculdade de Agronomia 20: 232-237.

Morris WL, Ducreux L, Griffiths DW, Stewart D, Davies HV and Taylor MA (2004) Carotenogenesis during tuber development and storage in potato. J Exp Bot 55(399): 975-982.

Morton AG (1981) History of botanical science: An account of the development of botany from ancient times to the present day. Academic Press: London.

Moutinho ILD, Bertges LC and Assis RVC (2007) Prolonged use of the food dye tartrazine (FD&C yellow nº5)and its effects on the gastric mucosa of Wistar rats. Braz J Bio 67(1): 141-145.

Murmann JP (2000) Knowledge and competitive advantage in the synthetic dye industry, 1850-1914: the coeveolution of firms, technology and national institutions in Great Britain, Germany and the United States. Business History Conference (Dec 2000). Enterprise & Society 1: 699-704.

Nagegowda DA (2010) Plant volatile terpenoid metabolism: Biosynthetic genes, transcriptional regulation and subcellular compartmentation. FEBS Lett 584: 2965-2973.

Nakamura Y, Tsuji S and Tonogai Y (2003) Analysis of proanthocyanidins in Grape Seed Extracts, Health Foods and Grape seed oils. J Health Sci 49(1): 45-54.

Natural History Museum (2007) Sweet Potato. Retrieved on 7 July 2010, from http://www.nhm.ac.uk/jdsml/nature-online/seeds-of-trade/

Neudert U, Martínez-Férez IM, Fraser PD and Sandmann G (1998) Expression of an active phytoene synthase from *Erwinia uredovora* and biochemical properties of the enzyme. Biochimica et Biophysica Acta 1392: 51–58.

Ng JH and Tan B (1988) Analysis of palm oil carotenoids by HPLC with diode-array detection. J Chromatogr Sci. 26(9): 463-9.

Nissila EAJ, Rao RV, Engelmann F and Riley KW (1999) Ex situ strategies for complementary conservation of Asian sweet potatoes. Plant Genetic Resources Newsletter No. 117: 1-11.

Nonier MF, De Gaulejac NV, Vivas N and Vitry C (2004) Characterization of carotenoids and their degradation products in oak wood. Incidence on the flavour of wood. CR Chimie 7: 689–698.

Novefeel (2005) Plant Secondary Metabolites. Retrieved on 6 July 2010, from http://www.novafeel.com /nutrition/plant-secondary-metabolites.htm

Ogura N (1972) Studies on chlorophyllase in tea leaves. III. Properties of soluble and insoluble chlorophyllases. Plant Cell Physiol 13: 971-979.

Ojha A, Yaduvanshi SK and Srivastava N (2011) Effect of combined exposure of commonly used organophosphate pesticides on lipid peroxidation and antioxidant enzymes in rat tissues. Pesticide Biochem Phys 99(2): 148-156.

Olmedilla B, Granado F, Blanco I and Vaquero M (2003) Lutein: but not α-tocopherol, supplementation improves visual function in patients with age-related cataracts, a 2-y double blind placebo-controlled pilot study. Nutr 19: 21-24.

Ostlund Jr RE (2002) Phytosterols in human nutrition. Annu Rev Nutr 22: 533-549.

Osto LD, Lico C, Alric J, Giuliano G, Havaux M and Bassi R (2006) Lutein is needed for efficient chlorophyll triplet quenching in the major LHCII antenna complex of higher plants and effective photoprotection *in vivo* under strong light. Biomed Central Plant Bio 6: 32.

Ostrakhovitch EA and Afanas'ev IB (2001) Oxidative stress in rheumatoid arthritis leukocytes: suppression by rutin and other antioxidants and chelators. Biochem Pharm 62: 743-746.

Overstreet DH (2000) Organophosphate pesticides, cholinergic function, and cognitive performance in advanced age. Neurotox 21**,** 75–81.

Oyaizu M (1986) Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine. Jpn J Nutr 103: 413-419.

Papas AM (2002) Beyond α-tocopherol: the role of the other tocopherols and tocotrienols, In Meskin MS, Bidlack WR, Davies AJ and Omaye ST, Phytochemicals in Nutrition and Health. CRC press: New York. Pp 61–78

Paschold A, Halitschke R and Baldwin IT (2006) Using 'mute' plants to translate volatile signals. Plant J l45: 275–291.

Perry A, Rasmussen H and Johnson EJ (2009) Xanthophyll (lutein, zeaxanthin) content in fruits, vegetables and corn and egg products. J Food Comp Anal 22: 9-15.

Pesek CA and Warthesen JJ (1987) Photodegradation of carotenoids in a vegetable juice system. J Food Sci 52: 744–746.

Pesek CA and Warthesen JJ (1988) Characterization of the photodegradation of β-Carotene in aqueous model systems. J Food Sci 53: 1517–1520 .

Pesek CA, Warthesan JJ and Taoukis PS (1990) A kinetic model for equilibration of isomeric β-carotene. J Agric Food Chem 38: 41-45.

Pfander H, Lanz M and Traber B (1998) Synthesis of Carotenoids. In: Rahman AR (1998) Studies in Natural Products Chemistry. Volume 20: Structure and Chemistry (Part F). Elsevier: The Netherlands. Pp 613-658.

Pierre M, Traverso JA, Boisson B, Domenichini S, Bouchez D, Giglione C *et al* (2007) N-myristoylation reglates the SnRK1 pathway in Arabidopsis. Plant Cell 19: 2804-2821.

Podell S and Gribskoy M (2004) Predicting N-terminal myristoylation sites in plant proteins. BMC Genomics 5(1): 37.

Pop C, Ghibu GD, Gârban G and Velciov AB (2006) Structural peculiarities of carotenoids used as food colourants. J Agroalimentary Processes and Technologies (Review - Natural Food Extracts and Additives Section). 7(2): 461-468.

Porter NA, Mills KA and Caldwell SE (1995) Mechanisms of free radical oxidation of unsaturated lipids. Lipids 30: 277-290.

Pratheesh VB, Benny N and Sujatha CH (2009) Isolation, stabilization and characterization of xanthophyll from Marigold flower (*Tagetes Erecta* L.). Modern Applied Science 3(2): 19-28.

Prior RL, Hoang H, Gu L, Wu X, Bacchiocca M, Howard L *et al*. (2003) Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC)) of plasma and other biological and food samples. J Agric Food Chem 51: 3273–3279.

Raffauf RF (1996) Plant alkaloids: A guide to their discovery and distribution. Hawkworth Press: New York.

Rao R and Campilan D (2001) Exploring the complementarities of in situ and ex situ conservation startegies for Asian sweetpotato genetic resources. Proceedings of the 3rd International Workshop of the Asian Network for Sweetpotato Genetic Resources (ANSWER). Denpasar, Bali, Indonesia, 2-4 October 2001.

Rayon C, Lerouge P and Faye L (1998) The Protein N-glycosylation in Plants. J Exp Bot 49(326): 1463-1472.

Reddy ASN (2007) Alternative splicing of pre-messenger RNAs in plants in the genomic era. Annu Rev Plant Biol 58:267–294.

Rimbach G, Fuchs J and Packer L (2005) Application of nutrigenomics tools to analyze the role of oxidants and antioxidants in gene expression, In: Rimbach G, Fuchs J and Packer L (eds.), Nutrigenomics. Taylor & Francis: Boca Raton, FL. Pp 1–12.

Rispail N, Morris P and Webb KJ (2005) Phenolics compounds: extraction and analysis. In Márquez AJ lotus japonicus handbook 349-355.

Rodriguez-Amaya DB (1997) Carotenoids and food preparation: The retention of provitamin A carotenoids in prepared, processed and stored foods. OMNI Project, SAID.

Rozzi NL, Singh RK, Vierling RA and Watkins BA (2002) Supercritical fluid extraction of lycopene from tomato processing by products. J Agric Food Chem 50: 2638–2643.

Ruiz ME, Pezo D and Martinez L (1980) The use of sweet potato (*Ipomoea batatas*. L) in animal feeding. Agronomic Aspects Tropical Animal Production 5: 144-151.

Sacks FM and Kass EH (1988) Low blood pressure in vegetarians: effects of specific food and nutrients. Am J Clin Nutr 48: 795-800.

Sadraei H, Ghannadi A and Malekshahi K (2003) Relaxant effect of essential oil of *Melissa officinalis* and citral on rat ileum contractions. Fitoterapia 74: 445-452.

Saito S, Okamoto Y and Kawabata J (2004) Effects of alcoholic solvents on antiradical abilities of protocatechuic acid and its alkyl esters. Biosci, Biotechnol, Biochem 68: 1221-1227.

Salah N, Miller NJ, Paganga G, Tijburg L, Bolwell GP and Rice-Evans CA (1995) Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. Arc Biochem Biophy 322: 339−346.

Salvayre AN, Dousset N, Ferretti G, Bacchetti T, Curatola G and Salvayre R (2006) Antioxidant and cytoprotective properties of high-density lipoproteins in vascular cells. Free Rad Bio Med 41(7): 1031-1040.

Sánchez JF, Fernández JM, Acién FG, Pérez J and Molina E (2007) Influence of culture conditions in the productivity and lutein content of the new strain Scenedesmus almeriensis. Process Biochem 43(4): 398–405.

Sánchez JF, Fernández-Sevilla JM, Acién FG, Cerón MC, Pérez-Parra J and Molina-Grima E (2008) Biomass and lutein productivity of *Scenedesmus almeriensis*: influence of irradiance, dilution rate and temperature. Appl Microbiol Biotechnol 79: 719–729.

Sandmann G (2002) Molecular evolution of carotenoid biosynthesis from bacteria to plants. Physio Plantarum 116: 431–440.

Sawa T, Nakao M, Akaike T, Ono K and Maeda H (1999) Akylperoxyl radical-scavenging activity of various flavonoids and other phenolic compounds: implicatins for the anti-tumor-promoter effect of vegetables. J Agric Food Chem 47: 397-402.

Schafer H and Wink M (2009) Medicinally important secondary metabolites in recombinant microorganisms or plants: progress in alkaloid biosynthesis. Biotechnol Journal 4(12): 1684-1703.

Schaffer S, Schmitt-Schillig S, Muller WE and Eckert GP (2005) Antioxidant properties of Mediterranean food plant extracts: Geographical differences. J Phy Phar 56(1), 115-124.

Schripsema J, Fung SY and Verpoorte R (1996) Screening of plant cell cultures for new industrially interesting compounds, In: DiCosmo F and Misawa M (eds.) Plant Cell Culture Secondary Metabolism. Toward Industrial Application. CRC Press: Boca Raton, Florida. Pp 1-10.

Schwartz SJ and Lorenzo TV (1991) Chlorophyll stability during continuous aseptic processing and storage. J Food Sci 56: 1059.

Seigler DS (1998) Plant secondary Metabolites. Springer.

Seo JS, Burrib BJ, Quan Z and Neidlinger TR (2005) Extraction and chromatography of carotenoids from pumpkin. J Chrom A 1073: 371–375.

Setiawan B, Sulaeman A, Giraud DW and Driskell JA (2001) Carotenoid Content of Selected Indonesian Fruits. J Food Comp Anal 14: 169-176.

Sharma P and Bhat TK (2009) DPPH antioxidants assay revisited. Food Chem 113: 1202-1205.

Sharpless KE, Arce-Osuna M, Thomas JB and Gill LM (1999) Value assignment of retinal, retinyl palmitate, tocopherol and carotenoid concentrations in standard reference material 2383 (Baby Food Composite). J AOAC International 82: 288–296.

Shewmaker CK, Sheehy JA, Daley M, Colburn S and Ke DY (1999) Seed specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. Plant J 20: 401-412.

Shi J, Arunasalam K, Yeung D, Kakuda Y, Mittal G and Jiang YM (2004) Saponins from edible legumes: chemistry, processing and health benefits. J Medicinal Food 7(1): 67-78.

Shon MY, Kim TH and Sung NJ (2003) Antioxidants and free radical scavenging activity of *Phellinus baumii* (*Phellinus* of *Hymenochaetaceae*) extracts. Food Chem 82: 593–597.

Sian NK and Ishak S (1991) Carotenoid and anthocyanin contents of papays and pineapple: influence of blanching and predrying treatments. Food Chem 39: 175-185.

Simkin AJ, Moreau H, Kuntz M, Pagny G, Lin C, Tanksley S *et al*.(2008) An investigation of carotenoid biosynthesis in *Coffea canephora* and *Coffea Arabica*. J. Plant Physiol 165(10): 1087-1106.

Skelton RL, Yu Q, Srinivasan R, Manshardt R, Moore PH and Ming R (2006) Tissue differential expression of lycopene beta-cyclase gene in papaya (Unpublished) Direct Submission to Genbank. Available at http://www.ncbi.nlm.nih.gov/protein/90186657?report=genbank&log$=prottop&blast_rank=4&RID=C11EMPF201S

Snodderly DM (1995) Evidence of protection against age-related macular degeneration by carotenoids and antioxidant vitamins. Am J Clinical Nutr 62: 1448S-1461S.

Sommer A (1995) Vitamin A Deficiency and Its Consequences: A Field Guide to Detection. World Health Organization, Geneva.

Spencer J (2007) China Pays Steep Price As Textile Exports Boom. The Wall Street Journal, August 22, 2007. Retrieved on 3 October 2011, from http://online.wsj.com /public/article/

Straub AM (2005) Colourful demands. The world of food ingredients. Apr/May 2005: 16-19.

Su Q, Rowley KG and Balazs NDH (2002) Carotenoids: separation methods applicable to biological samples. J Chrom B 781: 393–418.

Summers C, Karst F and Charles AD (1993) Cloning, expression and characterisation of the cDNA encoding human hepatic squalene synthase, and its relationship to phytoene synthase. **Gene 136: 185-192.**

Sun B, Ricardo-da-Silva JM and Spranger I (1998) Critical factors of vanillin assay for catechins and proanthocyanidins. J Agric Food Chem 46: 4267-4274.

Sun ZR, Gantt E and Cunningham FX Jr (1996) Cloning and functional analysis of the β-carotene hydroxylase of *Arabidopsis thaliana*. J Biol Chem 271: 24349-24352.

Tadeg H, Mohammed E, Asres K and Gebre-Mariam T (2005) Antimicrobial activities of some selected traditional Ethiopian medicinal plants used in the treatment of skin disorders. J Ethnopharmacol 100: 168-175.

Tai CY and Chen BH (2000) Analysis and stability of carotenoids in the flowers of Daylily (*Hemerocallis disticha*) as affected by various treatments. J Agric Food Chem 48: 5962-5968.

Takaichi S, Maoka T, Takasaki K and Hanada S (2010) Carotenoids of *Gemmatimonas aurantiaca* (*Gemmatimonadetes*): identification of a novel carotenoid, deoxyoscillol 2-rhamnoside, and proposed biosynthetic pathway of oscillol 2,29-dirhamnoside. Microbiology 156: 757–763.

Tan SL and Mohammad AA. Country report on the status of sweetpotato germplasm in Malaysia: collection and maintenance. Proceedings of the 3rd International Workshop of the Asian Network for Sweetpotato Genetic Resources (ANSWER). Denpasar, Bali, Indonesia, 2-4 October 2001.

Tanaka Y, Sasaki N and Ohmiya A (2008) Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. Plant J 54 (4): 733-749.

Tanganik M, Phezo P, Ewell PT, Lutaladio NB and Scott G (1999) The role of potato and sweetpotato in disaster relief: The case of Rwandan refugees in South Kivu, Democratic Republic of the Congo (ex-Zaire) 1994–96. In: Program Report, 1998-1999, International Potato Center (CIP). CIP, Lima, Peru.

Tao N, Xu J, Cheng Y, Guo W and Deng X (2004) Carotenoid biosynthesis in citrus. (Unpublished) Direct Submission to Genbank. Available at http://www.ncbi. nlm.nih.gov/protein/51511937?report=genbank&log$=prottop&bla st_rank=8&RID=C11EMPF201S

Tartrazine (2010) 30 years of Poisoning by the most common food additives. Retrieved on 21 March 2010, from http://www.natural-health-for-you.com/tartrazine .html

Taungbodhitham AK, Jones GP, Wahqvist ML and Briggs DR (1998) Evaluation of extraction method for the analysis of carotenoids in fruits and vegetables. Food Chem 63: 577-584.

Tee ES (1995) The medical importance of vitamin A and carotenoids (with particular reference to developing countries) and their determination. Malaysian J Nutr 1: 179-230.

The Pennsylvania State University (2009) Leaf stage and strategy. Retrieved on 30 March 2010, from http://www.psu.edu/dept/nkbiology/naturetrail/leaves.htm.

Tlili N, Nasri N, Saadaoui E., Khaldi A and Triki S (2009) Carotenoid and tocopherol composition of leaves, buds, and flowers of *Capparis spinosa* grown wild in Tunisia. J Agric Food Chem 57: 5381–5385.

Torrey G (2011) Improved nutrition may reduce the risk of macular degeneration. American Macular Degeneration Association. Retrieved on 18th October 2011, from http://www.macular.org/nutrition/index.html.

Towler DA, Adam SP, Eubanks SR, Towery DS, Jackson-Machelski E, Glaser L *et al.* (1988) Myristoyl CoA: Protein N-Myristoyltransferase activities from rat liver and yeast possess overlapping yet distinct peptide substrate specifities. J Bio Chem 263(4): 1784-1790.

Trujillo-Quijano J, Rodriguez-Amaya DB, Esteves W and Plonis GF (1990) Carotenoid composition and vitamin A values of oils from four Brazilian palm fruits. Eur J Lipid Sci Tech 92(6): 222-226.

Tsimidon M and Biliadesis CG (1993) Stability of saffron pigments in aqueous extracts. J Food Sci 45: 2890-2898.

Tsuda S, Murakami M, Matsusaka N, Kano K, Taniguchi K and Sasaki YF (2001) DNA damage induced by red food dyes orally administered to pregnant and male mice. Toxicological Sci 61(1): 92–99.

Tsuda T, Horio F, Kitoh J and Osawa T (1999) Protective effects of dietary cyaniding 3-O-b-D-glucoside on liver ischemia-reperfusion injury in rats. Arc Biochem Biophy 368: 361–366.

US-EPA (1997) Issues paper Guidance for establishing a common mechanism of toxicity for use in combined risk assessments. Presented to the F1FRA Scientific Advisory Panel March 20, 1997. US EPA, Washington DC.

US-EPA (2012) Pesticides and Food: What the pesticides residue limits are on food? Retrieved on 13 April 2012, from http://www.epa.gov/pesticides/food/viewtols.htm

Van het Hof KH, Brouwer IA, West CE, Haddeman E, Steegers-Theunissen RP, van Dusseldorp M, *et al*. (1999) Bioavailability of lutein from vegetables is 5 times higher than that of β-carotene. Am J Clinical Nutr 70: 261-268.

van Lieshout M, West CE, Permaesih D, Wang Y, Xu XY, van Breemen RB, *et al*. (2001) Bioefficacy of beta-carotene dissolved in oil studied in children in Indonesia. Am J Clinical Nutr 73: 949–958.

Verpoorte R (1998) Exploration of nature's chemodiversity: the role of secondary metabolites as leads in drug development. DDT 3(5): 232-238.

Verpoorte R, Contin A and Memelink J (2002) Biotechnology for the production of plant secondary metabolites. Phytochem Rev 1**:** 13–25.

Verpoorte R, van der Heijden R, ten Hoopen HJG and Memelink J (1999) Metabolic engineering of plant secondary metabolite pathways for the production of fine chemicals. Biotechnol Lett 21: 467–479.

Villareal RL (1982) Sweet potato in tropics: Progress and problems. In: Villareal RL and Griggs TD (Eds.). Proceedings of the 1st International Symposium on Sweet Potato. AVRDC, Taiwan, China, pp 3-15.

Walton NJ and Brown DE (1999) Chemicals from plants, perspectives on secondary plant products. Imperial College Press: United Kingdom. Pp 1-25.

Wan Q, Zhang XG and Song M (2007) Fruit-specific RNAi-mediated Lcy gene enhances content of lycopene in tomatoes silencing. Sheng Wu Gong Cheng Xue Bao 23(3): 429-433.

Wang D (2005) Sweet Potato Varietal Trail. ARC Traning.

Wang H, Cao G and Prior RL (1997) Oxygen radical absorbing capacity of anthocyanins. J Agric Food Chem 45: 304-309.

Wang L and Liu L (2009) Optimization of solvent extraction conditions for total carotenoids in rapeseed using response surface methodology. Nat Sci 1: 23-29.

Wang M, Xiong YH, Zeng MM, Li HD, Zhang TM and Liang YZ (2010) GC–MS combined with chemometrics for analysis of the components of the essential oils of sweet potato leaves. Chromatographia 71(10): 891-897.

Watada AE, Aulenbach, BB and Worthington JT (1976) Vitamins A and C in ripe tomatoes as affected by stage of ripeness at harvest and by supplementary ethylene. J Food Sci 41, 856-858.

Waterhouse AL (2002) Determination of Total Phenolics. Current Protocols in Food Analytical Chemistry I1.1.1- I1.1.8.

Watson RR and Preedy VR (2008) Botanical Medicine in clinical practice. Cromwell Press: United Kingdom. Pp 142-145.

Weisburger JH (1994) Does the Delaney Clause of the US Food and Drug Laws Prevents Human Cancers? Toxicological Sci 22(4): 483-493.

Werth G and Boiteaux A (1967) The toxicity of the triphenylmethane dyestuff malachite green, as an uncoupler of oxidative phosphorylation in vivo and in vitro. Arch Toxicol 23: 82–103.

Wettasinghe M and Shahidi F (1999) Antioxidant and free radical-scavenging properties of ethanolic extracts of defatted borage (*Borago officinallis L*.) seeds. Food Chem 67: 399–414.

Whisstock JC and Lesk AM (2003) Prediction of protein function from protein sequence and structure. Quarterly Rev Biophys 36(3): 307–340.

William III L and Martinson TE (2003) Nondestructive leaf area estimation of "Niagara" and "DeChaunac" grapevines. Scientia Hort 98: 493-498.

Williams S (1984)(Ed.) Official Methods of Analysis of the Association of Official Analytical Chemists (14th ed.). Pp 835.

Wills RBH and Rangga A (1996) Determination of carotenoids in Chinese vegetables. Food Chem 56(4): 451-455.

Wink M (1993) Allelochemical properties and the raison d'etre of alkaloids. In: Cordell G (ed.). The Alkaloids. Academic Press. Pp 1–118.

Wink M (1999) Biochemistry of Plant Secondary Metabolism. Annu Plant Rev (Vol. 2). Sheffield Academic Press: Sheffield.

Wink M (2000) Interference of alkaloids with neuroreceptors and ion channels. In: Atta-Ur-Rahman X (Ed.), Bioactive Natural Products. Elsevier. Pp 3–129.

Wink M (2003) Evolution of Secondary Metabolites from an Ecological and Morphological Phylogenetics Perspectives. Phytochem 64: 3-19.

Wink M and Schimmer O (1999) Modes of action of defensive secondary metabolites, In: Wink, E. (Ed.), Function of Plant secondary metabolites and their exploitation in biotechnology, Annu Plant Rev. Sheffield Academic Press and CRC Press. Pp 17–133.

Wink M and Waterman P (1999) Chemotaxonomy in relation to molecular phylogeny of plants. In: Wink, M. (Ed.), Biochemistry of plantsecondary metabolism, Annual Plant Reviews. Sheffield Academic Press and CRC Press. Pp 300–341.

Withy LM, Nguyen TT and Wrolstad RF (1993) Storage changes in anthocyanin concentrate of red raspberry juice content. J Food Sci 1: 190–192.

Wolfender JL, Ndjoko K and Hostettmann K. (2003) Liquid chromatography with ultraviolet absorbance–mass spectrometric detection and with nuclear magnetic resonance spectroscopy: a powerful combination for the on-line structural investigation of plant metabolites. J Chromatogr A 1000: 437-455.

Wolfender JL, Rodriguez S and Hostettmann K (1998) Liquid chromatography coupled to mass spectrometry and nuclear magnetic resonance spectroscopy for the screening of plant constituents. J Chromatogr A 794: 299-316.

Woolfe JA (1992) Sweet Potato: An untapped Resource Food. Cambridge University Press: United Kingdom. Pp 1-27.

Wu D and Simin NM (1999) Antioxidants and Immune Function, In: Papas AM (2nd ed.). Antioxidant Status, Diet, Nutrition and Health. CRC Press: New York. Pp 371 - 400.

Yamamizo C, Kishimoto S and Ohmiya A (2010) Carotenoid composition and carotenogenic gene expression during Ipomoea petal development. J Exp Bot 61(3): 709-719.

Ye X, Al Babili S, Kloti A, Zhang J, Lucca P, Beyer P, *et al*. (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287: 303-305.

Yen WJ and Chen BH (1995) Isolations of xanthophylls from Taiwanese orange peels and their effects on the oxidation stability of soybean oil. Food Chem 53: 417-425.

Yoo SD, Greer DH, Laing WA and McManus MT (2003) Changes in photosynthetic effiency and carotenoid compostion in leaves of white clover at different developmental stages. Plant Physio Biochem 41: 887–893.

Young A and Britton G (1990) Carotenoids and stress. In Stress Responses in Plants: Adaptation and Acclimation Mechanisms. Wiley-Liss: London.

Yun DJ, Hashimoto T and Yamada Y (1992) Metabolic engineering of medicinal plants; Transgenic *Atropa belladonna* with an improved alkaloid composition. Proc. Natl. Acad. Sci. USA 89: 11799–11803.

Zechmeister L and Sandoval A (1946) Phytofluene. J Am Chem Soc 68(2): 197-201.

Zelkha OM, Sedlov T and Sheva B (2004) Carotenoids extraction process. United States Patent US 6797303 B2.

Zheng W and Wang SY (2001) Antioxidant activity and phenolic compounds in selected herbs. J Agr Food Chem 49: 5165-5170

Zhu C, Yamamura S, Nishihara M, Koiwa H and Sandmann G (2003) cDNAs for the synthesis of cyclic carotenoids in petals of Gentiana lutea and their regulation during flower development. Biochim Biophys Acta-Gene Struct Expr 1625: 305–308.