THE OPTIMUM OPERATING CONDITIONS FOR EXTRACTION OF CHLOROPHYLL FROM *Alternanthera sessilis* (Linn.), CULTIVATED IN SRI LANKA

MASTER OF SCIENCE

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UNIVERSITY OF MORATUWA
SRI LANKA

SEPTEMBER 2010

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BY

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THIS THESIS WAS SUBMITTED TO THE
DEPARTMENT OF CHEMICAL AND PROCESS ENGINEERING
OF UNIVERSITY OF MORATUWA
IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE
DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF CHEMICAL AND PROCESS ENGINEERING
UNIVERSITY OF MORATUWA
MORATUWA
SRI LANKA

SEPTEMBER 2010

Declaration

The work submitted in this dissertation is the result of my own investigation, except otherwise stated. It has not already been accepted for any degree, and is also not being concomitantly submitted for any other degree or diploma in any other university. It does not contain any material previously published, written or orally communicated by another person, to the best of my knowledge and belief.

.....

MAM Jinasena

10th of September, 2010

We endorse the declaration by the candidate.

.....

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Acknowledgement

My profound gratitude and sincerest appreciation go to the supervisors, Dr. ADUS Amarasinghe, Prof. (Mrs.) BMWPK Amarasinghe and Dr. MAB Prashantha; head of the department, senior lecturer of the department (Dpt. of Chemical & Process Engineering) and lecturer of ITUM, University of Moratuwa, respectively, whose support was vital to this research. Their encouragement, guidance and expert help was invaluable.

The support given by the staff of the Post Graduate Studies Division and the Senate Research Grant was always encouraging. Special thanks go to Dr. S Walpolage and Dr. (Mrs.) SHP Gunawardena for their suggestions and the encouragement given as the members of the progress review panel. Gratitude goes to Dr. PG Rathnasiri, the course coordinator of 'MSc/PG Diploma in Sustainable Process Development', for allowing me to study the subject "Computational Fluid Dynamics" as my coursework component.

Appreciation is extended to Dr. J Manatunga, head of Environment Division, Dpt. of Civil Engineering, University of Moratuwa, and the technical staff of the Environmental Engineering Laboratories of both departments, Dpt. of Civil and Chemical Engineering, for the help given in the spectrophotometric analysis.

Critical help needed in laboratory work, was given by Ms. Amali Wahalathanthri, the laboratory technician of the Food Processing Laboratory of Dpt. of Chemical & Process Engineering. She went out of her way to help me to carry out the experiments and to gather the background knowledge. Also appreciated is the valuable support given by the academic and non- academic staff of the Dpt. of Chemical & Process Engineering.

Finally I would like to thank all the colleagues of the PG Division of the Dpt. of Chemical & Process Engineering and my family, for the help and cooperation given.

MAM Jinasena

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Abstract

Chlorophyll is widely extracted for industrial applications and is a key topic of scientific and commercial interest. *Alternanthera sessilis*, which is known as mukunuwenna in Sri Lanka, is one of best selections for the extraction of chlorophyll due to the good extraction efficiency, availability and low cost.

In this work, the optimum operating conditions for the extraction of chlorophyll from *A. sessilis* using solvent extraction were studied using buffered 80% aqueous acetone. The results revealed that the best solvent volume to *A. sessilis* weight ratio, which gives the highest yield of chlorophyll, was 5 ml/g. The effect of temperature and the extraction time on the extraction was also studied. The optimum temperature of extraction is 50°C and the optimum time of extraction is 45 minutes. The mass transfer of chlorophyll from *A. sessilis* at 20°C and 30°C was modeled mathematically, using general mass transfer equations. The experimental results showed that the degradation of chlorophyll beyond the temperature of 30°C is significant. The saturation solubility of chlorophyll *a* was 54.06 and 107.6µg/ml and that for chlorophyll *b* was 23.13 and 29.68 µg/ml at the temperatures of 20 and 30°C respectively.

Furthermore, the optimum pre-processing method and the storage conditions were studied as post harvest operations for *A. sessilis*. The optimum method of pre processing was identified as mechanical grinding. For one day storage, ambient air conditions (indoor) were the optimum conditions; and for a longer storage time it was the refrigerator conditions (15°C).

Using the extract, commercial chlorophyll, Sodium copper chlorophyllin has produced with a 33.3% conversion of chlorophyll and its derivatives, using a modified process. The kinetics of degradation of chlorophyll of the produced sodium copper chlorophyllin follows a first order relationship between the concentration and the time. The relationship between the rate constant and the temperature follows the Arrhenius behavior. The activation energy for chlorophyll *a* and *b* are 3.014 and 2.78 kcal/mol respectively. The rate constants for chlorophyll *a* and *b* at different temperatures are also obtained.

1 Introduction

This chapter includes a preface of the work, the objectives, scope and limitations of the study.

1.1 General

Chlorophyll is the green pigment found in many plants and necessary for the process of photosynthesis. The production of chlorophyll from algae and higher plants is a key topic of scientific and commercial interest. Chlorophyll is widely extracted for industrial applications from higher plants and algae.

In the present study, *Alternanthera sessilis*, which is commonly known as mukunuwenna in Sri Lanka, was selected due to high yield and low cost. *A. sessilis* is a perennial plant and is consumed as a leafy vegetable.

Among various solvents such as acetone, methanol, ethanol and dimethylsulfoxide, 80% aqueous acetone was selected as the solvent for this research since it produces more stable product, cheaper and is less toxic.

Present work determines the optimum operating conditions for the extraction of chlorophyll from *A. sessilis* to produce chlorophyll locally using readily available, low cost raw materials. Post harvesting operations for *A. sessilis* are also considered. Generally high yield are achieved with high temperature extractions but degradation can take place at high temperatures. Therefore the effect of temperature on the yield of chlorophyll during the extraction is examined.

Production of the most common type of commercial chlorophyll, sodium copper chlorophyllin is done using a modified process. Since the temperature and time is critical controllers of the quality of the product, the kinetics of chlorophyll is scrutinized.

1.2 Objectives

Objectives of the present work are,

1. To study the optimum operating conditions of extraction of chlorophyll from *A. sessilis*
2. To determine the optimum post harvesting methods of *A. sessilis*
3. To produce commercial chlorophyll and to study its stability

1.3 Scope

1. Only the most important operating conditions are studied.
2. The study was limited to the commercially available raw materials. The effect of varieties of *A. sessilis* was not included.
3. Analysis of chlorophyll was done by physical methods.

2 Literature Review

This chapter contains the essence of the literature review done. It includes the pre-requisite understanding of the work; basic information on chlorophyll, *Alternanthera sessilis*, quantification and degradation of chlorophyll as well as the derivatives of chlorophyll.

2.1 What is Chlorophyll?

Chlorophyll is the green pigment found in many plants, algae and cyanobacteria. It is formed in the chloroplast of the plant cell usually under the influence of sunlight, and necessary for the process of photosynthesis.

Chlorophyll has a complex and fascinating molecular structure (Fleming 1972), but differs from many of the complex natural substances. Chlorophyll is a chlorin pigment, which is structurally similar to porphyrin pigments. At the center of the chlorin ring is a magnesium ion; as shown in Figure 2-1. This has various side chains, usually including a long phytol chain. There are few different forms of chlorophyll that occur naturally. Some of the forms are shown in Table 2-1.

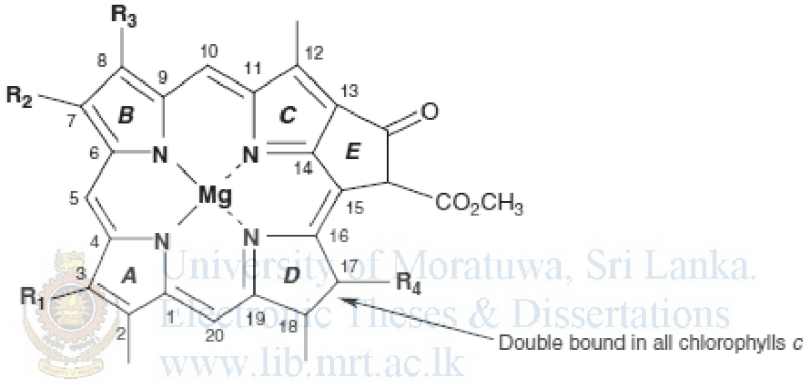

Figure 2-1 - Molecular structure of chlorophylls (Marquez 2009)

Table 2-1 – Some of the types of chlorophyll that occurs naturally

	Chlorophyll *a*	**Chlorophyll *b***	**Chlorophyll c_1**	**Chlorophyll *d***
Molecular Formula	$C_{55}H_{72}O_5N_4Mg$	$C_{55}H_{70}O_6N_4Mg$	$C_{35}H_{30}O_5N_4Mg$	$C_{54}H_{70}O_6N_4Mg$
R_1	$CH=CH_2$	$CH=CH_2$	$CH=CH_2$	CHO
R_2	CH_3	CHO	CH_3	CH_3
R_3	CH_2CH_3	CH_2CH_3	CH_2CH_3	CH_2CH_3
R_4	$(CH_2)_2$-COO-$C_{20}H_{39}$	$(CH_2)_2$-COO-$C_{20}H_{39}$	CH_2=CH-COOH	$(CH_2)_2$-COO-$C_{20}H_{39}$
Occurrence	Universal	Mostly in land plants	Various algae	Some red algae

Since most widely distributed forms in the terrestrial plants are chlorophyll *a* and *b*, the study was narrowed to those two forms. Chlorophyll is present in all plants, even though the content may vary depending on species, plant parts or growth stage. Chlorophyll is abundant in leafy vegetables and generally to a lesser extent in fruits. For example in leafy vegetables, chlorophyll can be as high as 1% on a dry weight basis.

2.2 Uses of Chlorophyll

Chlorophyll adds an important aesthetic dimension to the world we see, although it is so common that it may often go unnoticed. But it has the singular merit that, besides being beautiful, it is essential.

As the photosynthetic pigment, chlorophyll is directly responsible for the production of much of the food that we eat, and for all of the oxygen that we breathe.

Since the molecular structure of chlorophyll is very much similar to hemoglobin (except for the center atom), it helps in rebuilding and replenishing the red blood cells, boosting our energy and increasing our physical condition. Further, it helps improving various anemic conditions, improving the action of the heart, reducing abnormally high blood pressure and improving one's overall health (Kephart 1955). The late Dr. Bernard Jensen has discussed the uses of chlorophyll in detail in his book "The Healing Power of Chlorophyll." Some of the remedial effects of chlorophyll which he points out are listed below (Jensen 1973).

Builds a high blood count

Provides iron to the organs

Counteracts toxins eaten

Improves anemic conditions

Cleans and deodorizes bowel tissues

Helps purify the liver

Aids hepatitis improvement

Feeds iron to the heart

Regulates menstruation

Aids hemophilia conditions

Improves blood sugar problems (diabetes)

Aids in asthma improvement

Increases iron content in mother's milk

Improves mother's milk production

Helps sores heal faster

Eliminates body odors

Resists bacteria in wounds

Cleans tooth and gum structure in pyorrhea

Improve nasal drainage

Slows nasal drip

Lessens need for underarm deodorizers

Eliminates bad breath

Relieves sore throat	Aid catarrhal discharges
Makes an excellent tooth surgery gargle	Revitalizes vascular system in the legs
Benefits inflamed tonsils	Improves varicose veins
Soothes ulcer tissues	Reduces pain caused by inflammation
Soothes painful hemorrhoids and piles	

The deodorizing effect of chlorophyll makes it in the use of deodorizers and household fresheners (Taber 1951). Derivatives of chlorophyll can be used in wound treatments (Sack *et al.* 1955). Water soluble chlorophyllins can be used as therapeutic agents (Smith 1944). Furthermore the anti-mutagenic and anti-carcinogenic activities of chlorophyll and its derivatives (especially copper chlorophyllin) have been proved. Chlorophyll and its derivatives have anti-oxidant, anti-atherogenic, anti-inflammatory and detoxification properties. These are only a few of the multitude benefits that chlorophyll can do to the body. Chlorophyll is used in medicines and food supplements due to the above mentioned properties (Lai *et al.* 1980, Chernomorsky *et al.* 1999, Fernandes *et al.* 2007, Ferruzzi *et al.* 2007). Chlorophyll based fuel additives are used to enhance the combustion characteristics of carbonaceous fuels (Jordan 1998).

Their use as colorants is highly restricted due to their chemical instability and high production costs. Direct consumption of chlorophyll is associated with reduced risks of certain types of cancers. Researches on animals have shown that chlorophyll derivatives, such as chlorophyllin, exhibit antioxidant activity, at least as good as vitamin C. The function of chlorophyll in animals is suggested to be inhibition of lipid peroxidation and protection of mitochondria from oxidative damage induced by various free-radicals and other reactive oxygen species. Chlorophyll and chlorophyll derivatives are widely used in the industry as a stable, non-toxic, physiologically harmless colorant of dairy products, edible oils, soups, chewing gums, sugar confections, drinks, cosmetics, toiletries and medicines (Marquez *et al.* 2009).

The only side-effect of chlorophyll which have been reported in anecdote or scientific literature is the staining of skin or clothing (Golden *et al.* 1956).

2.3 *Alternanthera sessilis* (Linn.) R. Br. ex DC.

Various types of plants are used to extract chlorophyll around the world. Among these; spinach, alfalfa, algae, lichens, mallow, beans, soybean are common (Argyroudi-Akoyunoglou *et al.* 1970, Barnes *et al.* 1992, Simon *et al.* 1998, Fernandes *et al.* 2007).

In this study the source for the extraction of chlorophyll is selected as *Alternanthera sessilis* which is commonly known as 'mukunuwenna' in Sri Lanka, due to the availability and low cost in Sri Lanka and higher yield. *A. sessilis*, which is a perennial plant, is widespread throughout tropics and subtropics. It occupies moist areas and can be found from sea level to over 2000m. Although this plant is considering as a weed in other countries, Asians use this as a leafy vegetable. It has been used widely around the world for its medicinal uses, as well as for food.

Figure 2-2 – Parts of an A. sessilis plant

source (Levrault 1816-1829)

The plant has been readily used for food, due to its nutritional value and the abundance. In Sri Lanka, A. *sessilis* is also used in Aurvedic medicines. *A. sessilis* is used for the treatment of biliousness, chronic congestion of liver, acute and chronic pyelitis, cystitis. Further it increases the production of milk in nursing mothers (Jayaweera 1981). Recent researches showed the *A. sessilis* extract can prevent oxidative damage during storage of heated oil (Shyamala *et al.* 2005).

Along with other therapeutic applications, *A. sessilis* is used for diseases due to vitiated blood and obstinate skin diseases. The plant has stigmasterol, β-sitosterol, a saturated aliphatic hydrocarbon and aliphatic ester (Khare 2007). It is also used as a topical treatment for the common skin problem cane vulgarism. The antioxidant carotene is

found in large amounts in the plant (Jerajani *et al.* 2004). *A. sessilis* has used for the treatment of biliousness, dyspepsia associated with sluggish liver, chronic congestion of liver, acute and chronic pyelitis, cystitis, gonorrhea, and strangery and snake bite in Sri Lanka (Gayathri *et al.* 2006). In India and Sri Lanka, it is used for treatment of gastrointestinal problems. Further it is used as a treatment for headaches and vertigo in Nigeria and to treat hepatitis, bronchitis, and asthma in Taiwan (Jansen 2004). It is also popular due to its low cost and taste by the people of Sri Lanka (Gayathri *et al.* 2006). *A. sessilis* is chopped up when it is fed to ducks and is also fed to pigs (Ogle *et al.* 2003). It is used in many different foods in Africa, such as soups, relish, and sauces. *A. sessilis* can be dispersed through horticultural activities (Maki *et al.* 2004).

The scientific classification of *A. sessilis* is included in the Appendix.

2.4 Quantification of Chlorophyll

The most frequent practice to measure the amount of individual chlorophylls is spectrophotometry in mixed extracts rather than separate them by HPLC which is both costly in time and materials and often difficult to correct for measurements at a single wavelength and for losses during the extract manipulations. The much quoted equations of Arnon (1949) to determine individual levels of chlorophyll *a* and *b* in 80% (v/v) acetone in water are still used by many researchers. Arnon's equations were originally derived from the specific absorption (extinction) coefficients of Mackinney, (1941) using the 80% acetone as the solvent and always underestimate the ratio of chlorophyll *a* to *b*. It is inaccurate due to several reasons (Wellburn 1994), including the poor resolution of the spectrophotometers of the 1940s but the main problem is the solvent itself. The solvent does not extract all the major pigments completely and some of the less polar chlorophyll *a* and β carotene are always left behind especially in fibrous and other difficult plant tissues (Lichtenthaler 1987). Apart from that, efforts to improve the equations derived for 80% acetone have been made later including a correction for Arnon's chlorophyll *a*/*b* ratios (Vernon 1960, Ziegler *et al.* 1965, Lichtenthaler *et al.* 1983, Inskeep *et al.* 1985, Lichtenthaler 1987, Porra *et al.* 1989, Barnes *et al.* 1992).

The use of alternative solvents such as diethyl-ether, methanol, ethanol, dimethyl-formamide and DMSO has been prompted later (Moran *et al.* 1980, Moran 1982, Sartory *et al.* 1984, Inskeep *et al.* 1985, Porra *et al.* 1989, 1990, Barnes *et al.* 1992, Simon *et al.* 1998). There is a general agreement that the α coefficients of chlorophyll *a* and *b* derived by Smith *et al.* (1955) for diethyl-ether (ultra pure) are accurate and all other α coefficients on other solvents should be derived relative to the values in the manner

fully described by Porra *et al.,* 1989 (Wellburn 1994). None of the above researches except Porra *et al.,* 1989 have used the approved procedure. So considering all these facts Wellburn, 1994 has re-estimated the specific absorption coefficients for individual carotenoids and chlorophylls *a* and *b* using six solvents (80% acetone, chloroform, diethyl-ether, dimethylformamide, dimethylsulfoxide and methanol) using two different types of spectrophotometers (0.1-0.5 nm and 1-4 nm band pass resolution). From these values two sets of equations to calculate concentrations of chlorophyll *a* (C_a) and chlorophyll *b* (C_b) in µg/ml for the different instrument types were freshly derived or confirmed from earlier publications (Wellburn 1994). Considering the above results, 80% acetone was selected as the solvent since it is cheaper and is less toxic than the other solvents and the product is comparatively stable (Thompson *et al.* 1999). Therefore the Equation *2-1* and 2-2 is selected for the quantification of chlorophyll *a* and *b* with a resolution range of 0.1-0.5 nm of the spectrophotometer.

$$C_a = 12.25A_{663.2} - 2.79A_{646.8} \qquad 2\text{-}1$$

$$C_b = 21.5A_{646.8} - 5.1A_{663.2} \qquad 2\text{-}2$$

Where;

$A_{646.8}$ and $A_{663.2}$ are the absorbance at wave lengths of 646.8 and 663.2 nm, respectively.

2.5 Degradation of Chlorophyll

The degradation is the major quandary in the process of production of chlorophyll. Chlorophyll degradation is identified with leaf senescence. Yet it is not decided the pathway of degradation of chlorophyll during the extraction, the enzymatic breakdown of chlorophyll in leaf senescence has been established in recent years (Krautler 2002, Hortensteiner 2006, Krautler *et al.* 2006).

Senescence is the final stage of leaf development, which is followed by the death of the whole leaf. Chlorophyll is degraded in a pathway that is probably active in all higher plant species (Krautler *et al.* 1999, Pruzinska *et al.* 2003, 2005) and that leads to the formation of linear tetrapyrrolic breakdown products (nonfluorescent chlorophyll catabolites [NCCs]) that are deposited in the vacuoles of senescing cells.

The degradation occurs at several steps. The initialization of the 1st step is the removal of pytol chain in the presence of chlorophyllase to form chlorophyllide (Matile *et al.* 1996). Then the Magnesium ion is removed from the chlorophyllide by Mg - dechelatase

resulting pheophorbide (Langmeier *et al.* 1993, Suzuki *et al.* 2002). Then it is converted to pyropheophorbide by phaeophorbidase. (Matile *et al.* 1996, Takamiya *et al.* 2000) followed by the conversion in to primary fluorescent chlorophyll catabolite (pFCC) (Muhlecker *et al.* 1997). The latter conversion has been attributed to the action of two enzymes, pheophorbide *a* oxygenase (PAO) and red chlorophyll catabolite reductase (RCCR) (Hortensteiner *et al.* 1995, Rodoni *et al.* 1997), with red chlorophyll catabolite (RCC) occurring as an intermediate (Pruzinska *et al.* 2005). This is the 1st step of the pathway and a flow diagram is shown in Figure 2-3.

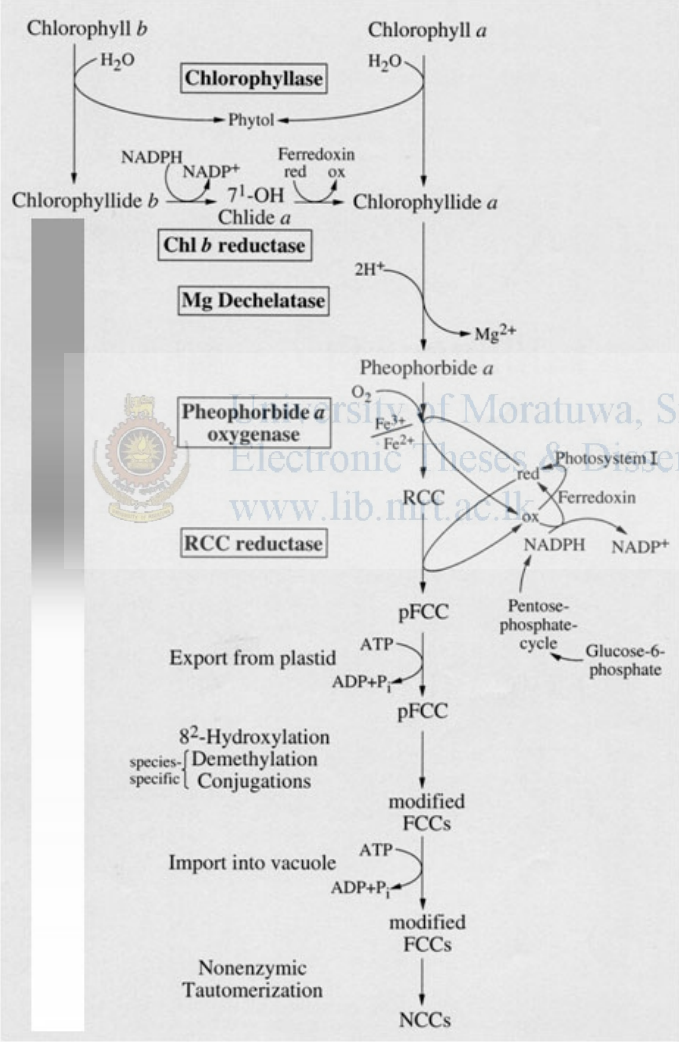

Figure 2-3 – The 1st step of the pathway of breakdown of chlorophyll

Image source- http://www.sidthomas.net/SenWiki/tiki-index.php?page_ref_id=159 accessed on 12th Nov. 2009

The rate of degradation of chlorophyll *b* is relatively low with compared to that of chlorophyll *a*. Further, it has been suggested that chlorophyll *b* enters the degradation pathway after it is converted to chlorophyllide *b* by chlorophyllase. Chlorophyllide *b* is then converted to chlorophyllide *a* by chlorophyll *b* reductase (Ito *et al.* 1996, Takamiya *et al.* 2000).

The molecular structures of the catabolites of the 1st step of the pathway of breakdown of chlorophyll are shown in Figure 2-4.

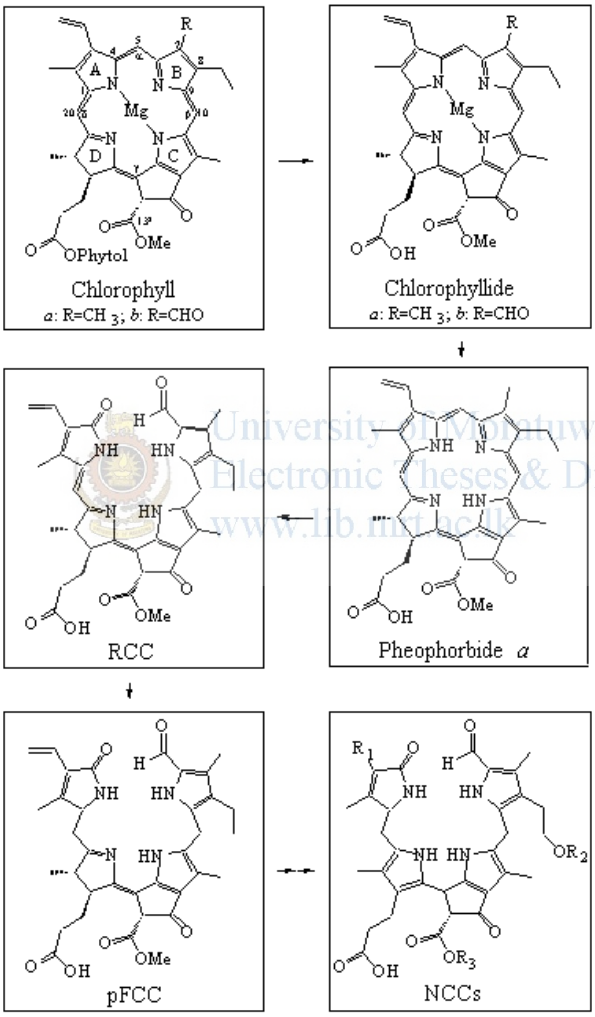

Figure 2-4 - The molecular structures of the catabolites of the 1st step

Image source- http://www.sidthomas.net/SenWiki/tiki-index.php?page_ref_id=159 accessed on 12th Nov. 2009

2.6 Production of Sodium Copper Chlorophyllin

In practice natural chlorophylls are rarely used for industrial purposes due to several reasons. Firstly, carotenoids, phospholipids and various other substances are co-extracted, resulting in products with diversified composition which makes subsequent purification steps indispensable. Natural chlorophylls are highly unstable and subject to color changes, being sensitive against low pH, heat, light, oxygen and the simultaneous action of enzymes. Therefore, endogenous plant enzymes and extraction conditions employed can easily promote chemical modification on the sensitive chlorophylls, yielding unattractive brownish-green degradation products like pheophytins and pheophorbides. Consequently, the production costs considering the mentioned difficulties are very high and therefore application of natural chlorophylls is limited. To overcome some of these limitations, semi-synthetic, metal-chelated and water-soluble chlorophyll derivatives, called chlorophyllins, have been produced as promising alternatives to fulfill industrial needs with a higher stability (Marquez *et al.* 2009).

The most popular commercial product of metal-chelated chlorophyllins corresponds to sodium copper chlorophyllin. It is prepared from a crude chlorophyll extract using solvent extraction and then chlorophyll is subjected to saponification allowing the hydro-phobic phytyl group to be displaced by sodium. After further fractionation and washing the chlorin salt can be marketed as the metal – free, grey-green water-soluble pigment. Conversion to a stable green colorant is achieved by acidification in the presence of copper salts (Lee *et al.* 1989, Hendry 1996).

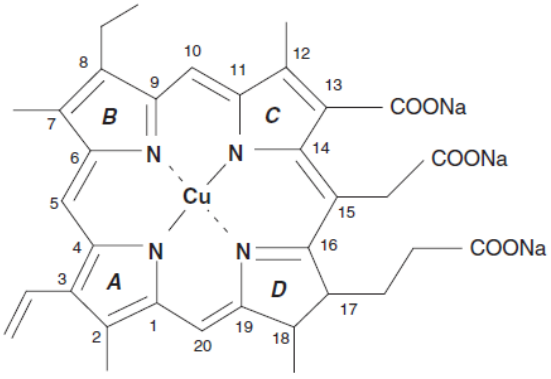

Figure 2-5 - Structure of trisodium copper chlorin e6

The substitution of Na by H at C-13 produces disodium copper chlorin e4. Both substances belong to the main constituents of commercial sodium copper chlorophyllin(Marquez 2009)

The common molecular structure of the commercially available sodium copper chlorophyllin is shown in Figure 2-5.

Although natural chlorophylls are highly unstable, the copper chlorophyllins are stable to moderate light and heat and are more resistant to low pH and oxidative reactions. For example, the fat soluble copper chlorophyllin was lost only to 42% after 500 h of exposure to light, while the magnesium chlorophyllin lost 95% during only 45 h of light exposure (Bobbio *et al.* 1990).

2.7 The methods of validation of a mathematical model

The following methods which are provided in Matlab™ itself have been used for the validation of the mathematical model which is developed to understand the effect of temperature on extraction, using general mass transfer theories.

- Residual analysis
- Confidence and prediction bounds
- 'Goodness of fit' (GoF) statistics

2.7.1 Residual analysis

The residuals from a fitted model are defined as the differences between the response data and the fit to the response data at each predictor value.

Residual = Observed data – Predicted data from model

$$r = y - \hat{y} \qquad 2\text{-}3$$

Assuming the model is correct, generally, the residuals approximate the random errors. Therefore, if the residuals appear to behave randomly, it suggests that the model fits the data well. However, if the residuals display a systematic pattern, it shows that the model does not fit the data (The MathWorks 2010).

2.7.2 Confidence and prediction bounds

In 'Curve Fitting Toolbox' software, confidence bounds can be calculated for the fitted coefficients, and prediction bounds for new observations or for the fitted function. The coefficient confidence bounds are shown numerically; while the prediction bounds are displayed graphically. Confidence and prediction bounds define the lower and upper

values of the associated interval, and define the width of the interval. The width of the interval indicates how uncertain are the fitted coefficients, the predicted observation.

2.7.2.1 Confidence bounds

The confidence bounds for fitted coefficients are given by

$$C = b \pm t\sqrt{S} \qquad 2\text{-}4$$

Where, b is the coefficients produced by the fit, t depends on the confidence level, and S is a vector of the diagonal elements from the estimated covariance matrix of the coefficient estimates, $(X^TX)^{-1}s^2$.

In a linear fit, X is the design matrix, while for a nonlinear fit X is the Jacobian of the fitted values with respect to the coefficients. X^T is the transpose of X, and s^2 is the mean squared error.

2.7.2.2 Prediction bounds

The prediction is based on an existing fit to the data. The non simultaneous prediction bounds for a new observation at the predictor value x are given by

$$P_{n,o} = \hat{y} \pm t\sqrt{s^2 + xSx'} \qquad 2\text{-}5$$

Where, s^2 is the mean squared error, t depends on the confidence level, and S is the covariance matrix of the coefficient estimates, $(X^TX)^{-1}s^2$. x is defined as a row vector of the design matrix or Jacobian evaluated at a specified predictor value.

The simultaneous prediction bounds for a new observation and for all predictor values are given by

$$P_{s,o} = \hat{y} \pm f\sqrt{s^2 + xSx'} \qquad 2\text{-}6$$

Where, f depends on the confidence level, and is computed using the inverse of the F cumulative distribution function (The MathWorks 2010).

2.7.3 'Goodness of fit' (GoF) statistics

Both the residual analysis and the analysis done by the use of 'confidence and prediction' bounds are graphical methods, while GoF statistics are numerical.

For parametric models, "curve fitting toolbox" software supports the following coefficients to determine the goodness of fit.

- SSE
- R^2
- Adjusted R^2
- RMSE

2.7.3.1 SSE – Sum of Squares due to Error

This statistic measures the total deviation of the response values from the fit to the response values. It is also called the summed square of residuals and is usually labeled as SSE.

$$SSE = \sum_{i=1}^{n} w_i \left(y_i - \hat{y}_i \right)^2 \qquad 2\text{-}7$$

A value closer to 0 indicates that the model has a smaller random error component, and that the fit will be more useful for prediction (The MathWorks 2010).

2.7.3.2 R^2 – R Square

R-square is the square of the correlation between the response values and the predicted response values. It is also called 'the square of the multiple correlation coefficient' and 'the coefficient of multiple determination'. R-square is defined as the ratio of the sum of squares of the regression (SSR) and the total sum of squares (SST).

SSR is defined as,

$$SSR = \sum_{i=1}^{n} w_i \left(\hat{y}_i - \bar{y} \right)^2 \qquad 2\text{-}8$$

SST is also called the sum of squares about the mean, and is defined as,

$$SST = \sum_{i=1}^{n} w_i \left(y_i - \bar{y} \right)^2 \qquad 2\text{-}9$$

Where, $SST = SSR + SSE$ *2-10*

Given these definitions, R-square is expressed as,

$$R-Square=\frac{SSR}{SST}=1-\frac{SSE}{SST} \qquad 2\text{-}11$$

R-square can take on any value between 0 and 1, with a value closer to 1 indicating that a greater proportion of variance is accounted for, by the model.

2.7.3.3 Adjusted R-Square - Degrees of Freedom

This statistic uses the R-square statistic defined in section 2.7.3.2, and adjusts it based on the residual degrees of freedom. The residual degrees of freedom is defined as the number of response values (n) minus the number of fitted coefficients (m) estimated from the response values.

$$v=n-m \qquad 2\text{-}12$$

v, indicates the number of independent pieces of information involving the n data points that are required to calculate the sum of squares.

$$Adjusted\ R-square=1-\frac{SSE(n-1)}{SST(v)} \qquad 2\text{-}13$$

The adjusted R-square statistic with a value closer to 1 indicates a better fit. Negative values can occur when the model contains terms that do not help to predict the response(The MathWorks 2010).

2.7.3.4 RMSE - Root Mean Squared Error

This statistic is also known as the 'fit standard error' and the 'standard error of the regression'. It is an estimate of the standard deviation of the random component in the data, and is defined as,

$$RMSE=s=\sqrt{MSE} \qquad 2\text{-}14$$

Where, $MSE$is the 'mean square error' or the 'residual mean square error' which is expressed as,

$$MSE=\frac{SSE}{v} \qquad 2\text{-}15$$

Just as with SSE, a $RMSE$ value closer to 0 indicates a fit that is more useful for prediction (The MathWorks 2010).

3 Materials and Methodology

This chapter describes the used resources and the methodology for the testing.

3.1 Materials and Chemicals

Matured and healthy *A. sessilis* samples were collected from a farm in Kesbewa, Sri Lanka. All parts of the plant except the roots were taken to prepare the samples. Oxidized copper wire particles were used for the copper replacement.

Double distilled acetone (SK laboratory (Pvt) Ltd, Colombo) was used as the solvent. Sodium di-hydrogen phosphate and disodium hydrogen phosphate were used to prepare a buffer solution of pH 7.8 and 2.5mM molarity.

A dual beam recording, UV-Visible spectrophotometer of model 1800 (Shimadzu™) with 1cm square glass cuvvetts was used for determination of chlorophyll concentration. This spectrophotometer allows automatic base line correction and data recording with more precise readings, and the resolution range is 0.1-0.5 nm. A vacuum filtration unit consists of a 5.5 cm diameter Whatman filter paper was used for filtration.

3.2 Methodology

80% (v/v) aqueous acetone was used as the solvent and 2.5 mM phosphate buffer at pH 7.8 was added to the solvent to control the pH variation.

For the experiments stated in sections 3.2.1, 3.2.2 and 3.2.3; fresh *A. sessilis* (5 g) was cut into pieces and ground mechanically into fine particles. The ground *A. sessilis* was added to the solvent and placed in a water bath. The extraction was carried out in a dark environment with frequent agitation.

After the extraction, samples were centrifuged for 10 minutes at 2,000 r.p.m. and the supernatant was separated for analysis of chlorophyll.

The solutions were diluted using the solvent, to obtain the absorbance values in the range of 0.1 to 0.7. The following equations were used for the determination of the concentrations of chlorophyll *a*, *b* and *a* plus *b* in diluted solutions, as stated in the section 2.4.

$$C_a = 12.25\, A_{663.2} - 2.79\, A_{646.8}$$

$$C_b = 21.5\, A_{646.8} - 5.1\, A_{663.2}$$

$$C_{a+b} = 7.15\, A_{663.2} + 18.71\, A_{646.8}$$

Where;

$A_{646.8}$ and $A_{663.2}$ are the absorbance at wave lengths of 646.8 and 663.2 nm, respectively. C_a and C_b are the concentrations of chlorophyll a and b in plant extract in micrograms per milliliter (µg/ml), respectively.

Concentrations of the extracts were calculated by multiplying the concentrations of diluted samples with the respective dilution factor. The weights of chlorophyll a/b were calculated by multiplying the concentrations of chlorophyll a/b with the volume of the sample.

Three replications were done for each experiment.

3.2.1 Determination of Optimum Solvent Volume to *A. sessilis* Weight Ratio

40g of *A. sessilis* was prepared as mentioned in section 3.2. The solvent volume to *A. sessilis* weight ratio was changed from 3 to 10 ml/g. The temperature of the water bath was kept at 30°C and the extraction time was 240 minutes. Concentrations and weights of chlorophyll *a*, *b* and *a* plus *b* were calculated.

3.2.2 Determination of Effective Temperature of Extraction

125g of *A. sessilis* was prepared and the solvent volume to *A. sessilis* weight ratio was 5ml/g. Five water baths were maintained at temperatures of 20, 30, 40, 50 and 60°C. Five samples were tested for each temperature. The extraction time was varied from 60 to 300 minutes with 60 minutes intervals. Concentrations and weights of chlorophyll *a*, *b* and *a* plus *b* were calculated.

3.2.3 Determination of Effective Time of Extraction

5g of *A. sessilis* was prepared as above keeping the water bath at a temperature of 50°C. The extraction time was varied from 15 minutes to 60 minutes with 15 minutes intervals, and then up to 300 minutes with 60 minutes intervals.

3.2.4 Effective Method of Pre Processing of *A. sessilis*

Five different pre- processing methods were used to disintegrate the structure of the plant cells.

3.2.4.1 Method 01

As the first method, fresh *A. sessilis* was cut and then ground for 5 minutes using grinder.

3.2.4.2 Method 02

Fresh *A. sessilis* was blanched by soaking the samples in hot water at a temperature of 95^{o}C for one minute and then immediately dipping it in cold water at a temperature of 15^{o}C for two minutes (Chandrika *et al.* 2006). Then the samples were cut into pieces of a length of one inch, approximately.

3.2.4.3 Method 03

Fresh *A. sessilis* was blanched following the procedure in section 3.2.4.2 and then ground according to the section 3.2.4.1.

3.2.4.4 Method 04

Fresh *A. sessilis* was ground using mortar and pestle.

3.2.4.5 Method 05

Fresh *A. sessilis* was dried in oven for six hours at a temperature of 40^{o}C.

Chlorophyll *a*, *b* and *a* plus *b* amounts were found for the determined optimum operating conditions tested above (Section 3.2.1, 3.2.2 and 3.2.3).

3.2.5 Effective Method of Storage Conditions for *A. sessilis*

The selected storage environments are refrigerator-freezer, refrigerator- cooler, the ambient air conditions-open, ambient air conditions- sealed, dried at 40^{o}C and dried at 70^{o}C. Fresh *A. sessilis* samples were kept in the selected environments and the recorded parameters are as shown in Table 3-1.

Each sample was checked daily for chlorophyll *a* and *b*. The storage time was limited to a maximum of 3 days. The determination of chlorophyll *a*, *b* and *a* plus *b* amounts were done in the same way as previous.

Table 3-1 - The tested environmental conditions

Environment	Average Temperature (°C) ±2	Average Relative Humidity % ±2	Light Condition
01	5	50	Enclosed
02	15	60	Enclosed
03	32	76	Open
04	32	76	Enclosed
05	40	76	Enclosed
06	70	76	Enclosed

3.2.6 Production of Sodium Copper Chlorophyllin

Fresh *A. sessilis* (10g) was cut into pieces and ground into fine particles. The ground *A. sessilis* was added to 50ml of the solvent and placed in a water bath at a temperature of 50°C for 45 minutes. The extraction was carried out in a dark environment with frequent agitation.

After the extraction, samples were centrifuged for 10 minutes at 2,000 r.p.m. and the supernatant was separated. Oxidized copper wire particles (5g) were added to the supernatant and the solution was heated at a temperature of 70°C until the acetone is evaporated. The residual liquid was removed using vacuum filtration. The solid was weighed and stored under room temperature. The produced sodium copper chlorophyllin was tested for chlorophyll *a* and *b*.

3.2.7 Study of the Kinetics of Degradation of Chlorophyll

4 samples of sodium copper chlorophyllin which is produced following the method in section 3.2.6 were kept at temperatures of 15, 30, 40 and 50°C. Each sample was tested for chlorophyll *a* and *b* for 0-6 hours at 1 hour intervals. Chlorophyll *a*, *b* and *a* plus *b* amounts were found for the determined optimum operating conditions tested in Section 3.2.1, 3.2.2 and 3.2.3. Each experiment was duplicated.

3.2.8 Development of Mathematical Model for the Mass Transfer of Chlorophyll

The mass transfer of chlorophyll from *A. sessilis* to the solvent was modeled as explained below.

When a material is being dissolved from the solid to the solvent solution, the rate of mass transfer from the solid surface to the liquid is the controlling factor. No resistance in the solid phase is assumed. The equation for the above process, is derived as follows (for a batch system) (Geankoplis 2002).

For the rate of mass transfer of the solute A being dissolved to the volume V m^3 is,

$$\frac{\overline{N}_A}{A} = k_L\left(c_{AS} - c_A\right) \qquad 3\text{-}1$$

Where;

$\overline{N}_A$ - Rate of solute A dissolving to the solution (mol/s)

A - Surface area of particles (m^2)

k_L - Mass transfer coefficient (m/s)

c_{AS} - Saturation solubility of the solute A in the solution (mol/m^3)

c_A - Concentration of solute A at time t sec (mol/m^3)

The rate of accumulation of A in solution,

$$V\frac{dc_A}{dt} = \overline{N}_A = Ak_L\left(c_{AS} - c_A\right) \qquad 3\text{-}2$$

Integrating from $t = 0$ and $c_A = c_{A0}$ to $t = t$ and $c_A = c_A$,

$$\int_{c_A=c_{A0}}^{c_A} \frac{dc_A}{c_{AS} - c_A} = \frac{Ak_L}{V}\int_{t=0}^{t} dt \qquad 3\text{-}3$$

$$\frac{c_{AS} - c_A}{c_{AS} - c_{A0}} = e^{-\left(\frac{k_L A}{V}\right)t} \qquad 3\text{-}4$$

Since $c_{A0} = 0$;

$$c_A = c_{AS} - c_{AS} \times e^{-\left(\frac{k_L A}{V}\right)t} \qquad \textit{3-5}$$

Where, $\frac{k_L A}{V} = K$ is a constant as; all the samples were prepared from the same lot, the solvent volume was kept constant and the extraction process was carried out at a constant temperature.

Finally the mass transfer equation is simplified to,

$$c_A = c_{AS} \times \left(1 - e^{-Kt}\right) \qquad \textit{3-6}$$

4 Results and Discussion

This chapter includes the results obtained from the experiments which are described in chapter 3 and a detailed discussion based on the results.

4.1 Determination of Optimum Solvent Volume to *A. sessilis* Weight Ratio

The concentration and weight of chlorophyll *a*, *b* and *a* plus *b*, which were obtained at different solvent to *A. sessilis* ratios for an extraction time of 120 minutes and extraction temperature of 30°C, were studied.

The concentration of chlorophyll *a*, *b* and *a* plus *b* versus solvent to *A. sessilis* ratio were plotted as shown in Figure 4-1. Generally, when the solvent volume increases the rate of extraction of chlorophyll increases, then the solution gets saturated, therefore further extraction becomes limited. This behavior is clearly shown in Figure 4-1.

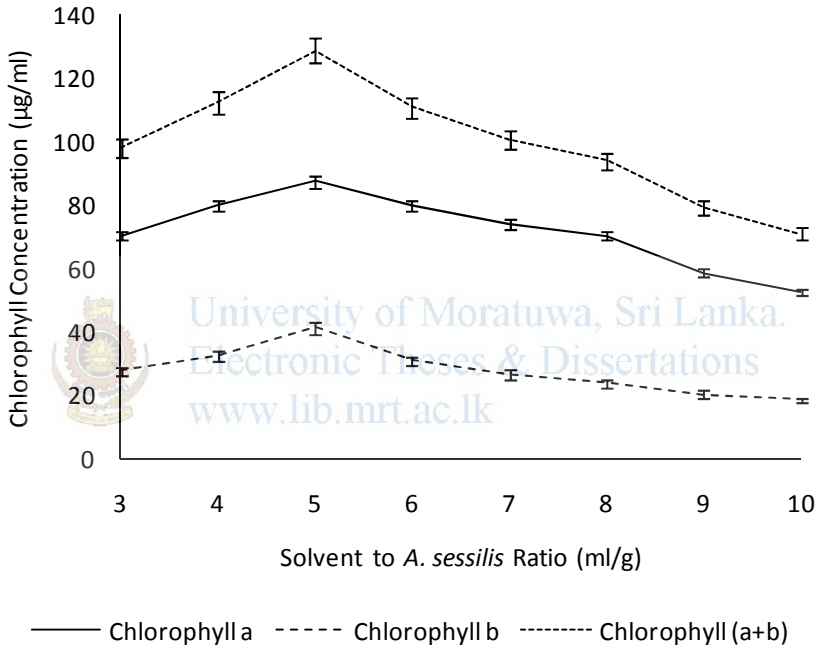

Figure 4-1 – Effect of solvent to A. sessilis ratio on chlorophyll concentration at 30°C

In all three types of chlorophylls, the concentration has increased up to ratio of 5. From ratio of 5 to 8, the concentration has reduced due to the dual effect of the increase of the rate of extraction and the dilution of the solution. Beyond the ratio of 8, the concentration has further reduced due to the dilution and the saturated condition of extraction.

The weight of chlorophyll *a*, *b* and *a* plus *b* versus solvent to *A. sessilis* ratio were plotted as shown in Figure 4-2. The weights of chlorophyll are independent of the solvent volume, and introduced on the base of 1g of *A. sessilis*.

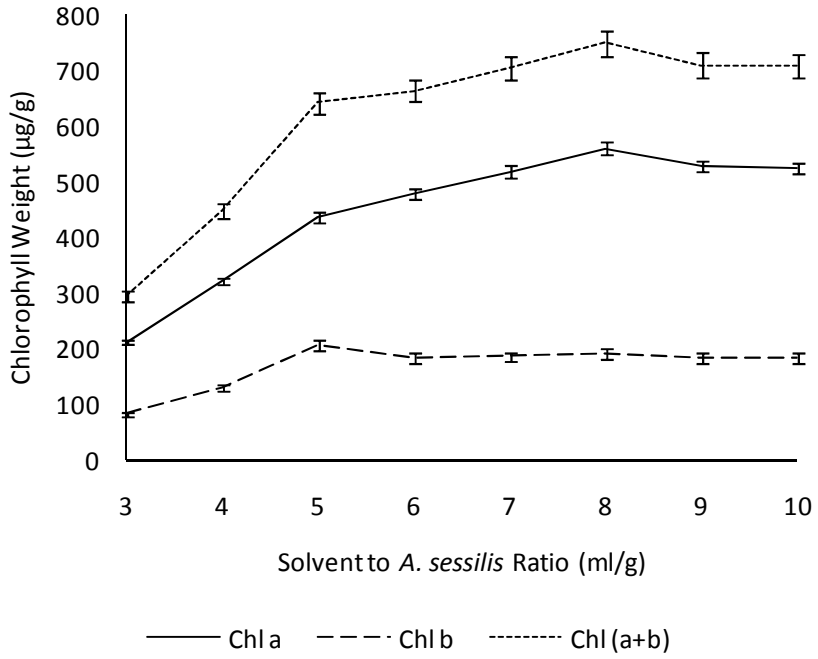

Figure 4-2 - Effect of solvent to A. sessilis ratio on chlorophyll weight at 30°C

The maximum weight is obtained at the ratio of 8. Although the weight is the highest, the solvent volume is higher at the ratio of 8 than at 5, and the increment of chlorophyll is less significant. Therefore the optimum ratio was selected as 5 as it is more economical.

The maximum chlorophyll concentration obtained was 128.7µg/ml. Meanwhile chlorophyll *a* and *b* concentrations were 87.4 and 41.3 µg/ml respectively, having a chlorophyll *a* to *b* ratio of 2.12 in the extract.

4.2 Determination of the Optimum Temperature of the Extraction

The concentration of chlorophyll *a*, *b* and '*a* plus *b*' in the extract with various extraction times for each temperature is shown in Figures Figure 4-3, 4-4 and 4-5, respectively. Since the highest chlorophyll concentration is obtained at the extraction temperature of 50°C, the optimum temperature is selected as 50°C.

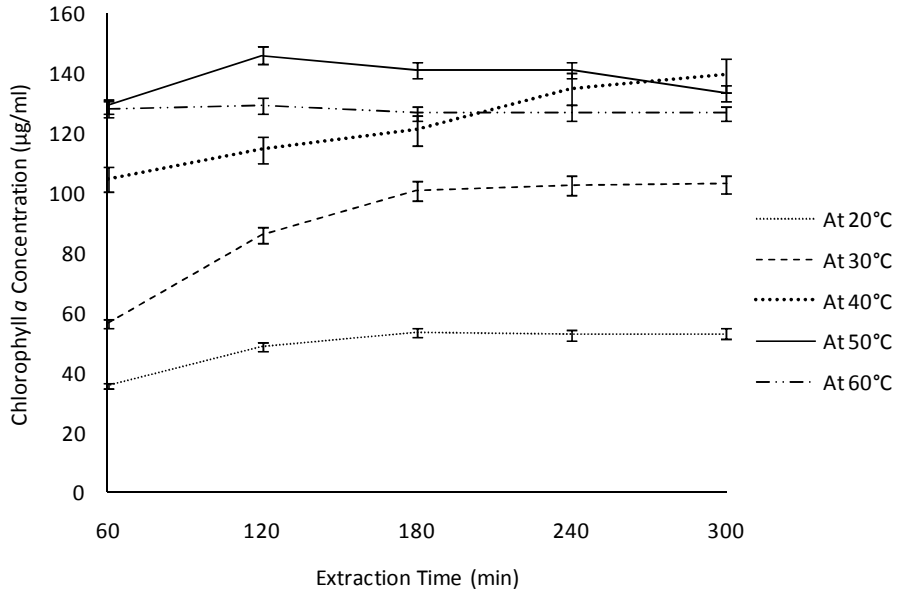

Figure 4-3 – Effect of extraction time and temperature on concentration of chlorophyll a

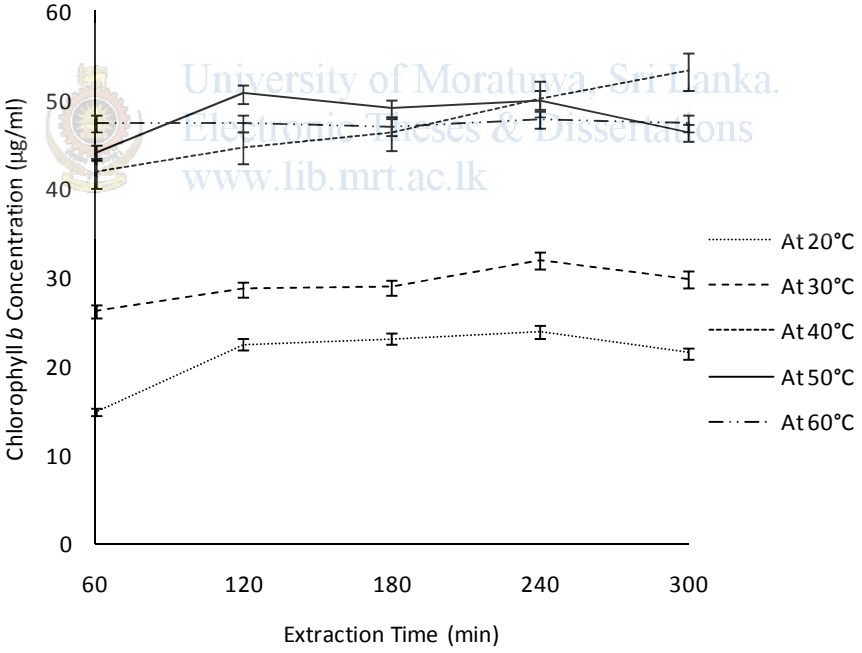

Figure 4-4 - Effect of extraction time and temperature on concentration of chlorophyll b

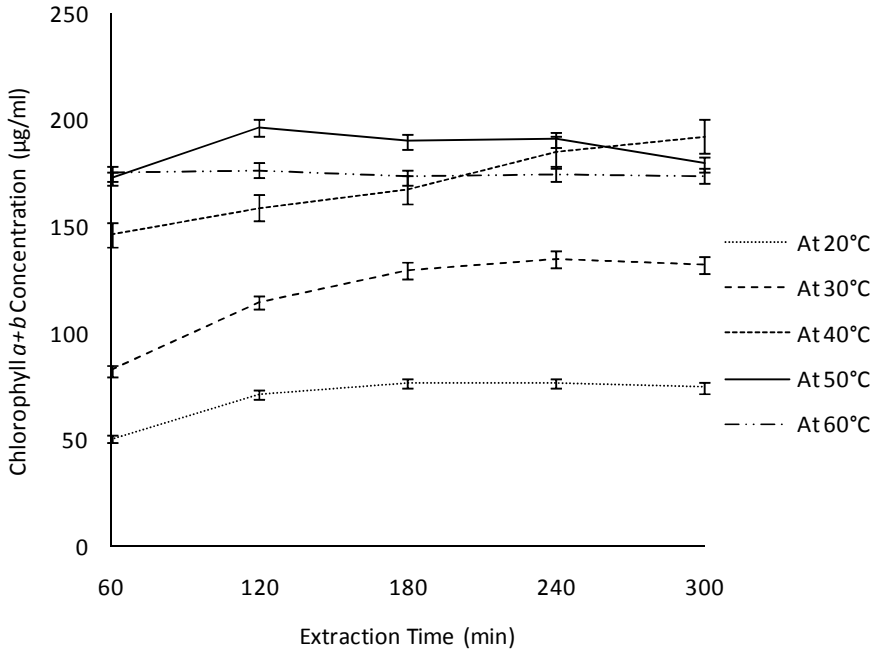

Figure 4-5 - Effect of extraction time and temperature on concentration of chlorophyll a and b

The observed behavior of the above figures is described at section 4.2.1 .

4.2.1 The Influence of the Temperature on the Extraction

Since the pattern of the variation of the concentration of chlorophylls has differed with the increase of temperature, the effect of temperature on the extraction was studied using the same results obtained from the experiments mentioned in section 3.2.2.

In Figure 4-5, three different patterns of the variation of concentration with the increase of temperature can be identified. At 20 and 30°C of temperature the concentration has increased with time up to 240 minutes and then has become a constant, while At 40°C of temperature the concentration has increased with time up to 300 minutes. But at 50 and 60°C of temperature the concentration has increased only up to 120 minutes and then it has decreased. It is suggested that the cause for this variation is the physical and chemical reactions that can be occurred and it is the extraction and the degradation of chlorophyll, respectively.

4.2.1.1 The Extraction – at Temperatures of 20 and 30°C

The concentration of chlorophyll *a*, *b* and *a* plus *b* at the temperatures of 20 and 30°C are shown in Figure 4-6. The general variation of concentration with time in an extraction is clearly observed.

Initially the concentration increases with the increase of extraction time, up to the saturated concentration and then becomes steady. This figure clearly indicates that the yield of chlorophyll had increased with the increase of temperature. The concentration of chlorophyll *a* : *b* ratio was found to be around 3.

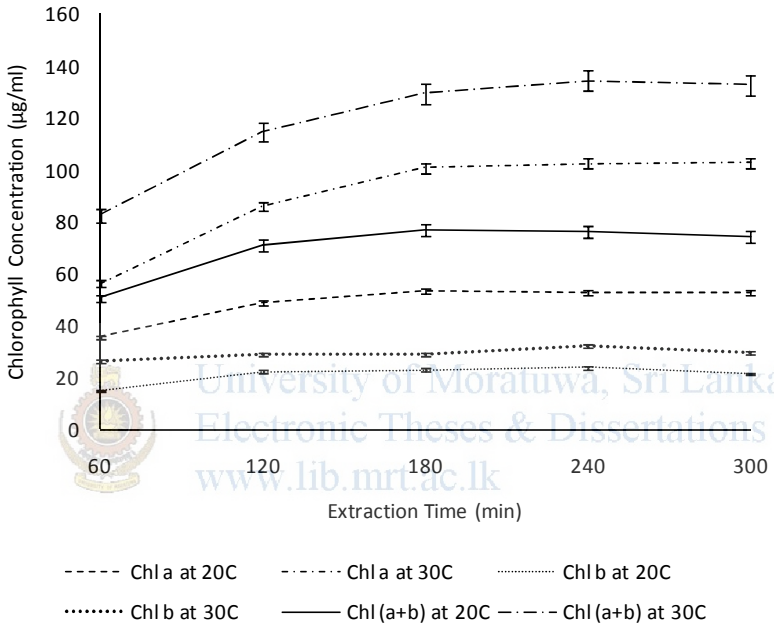

Figure 4-6 – Variation of chlorophyll concentration with time at 20 and 30°C

4.2.1.2 The Extraction with the Initialization of Degradation – at the Temperature of 40°C

The concentration of chlorophyll *a*, *b* and *a* plus *b* at the temperature of 40°C with the extraction time are shown in Figure 4-7.

Unlike the general mass transfer condition, a slight increase in concentration could be observed at this temperature, after about 180 minutes of extraction. This can be attributed to a double effect of the increase in extraction and the degradation of chlorophyll.

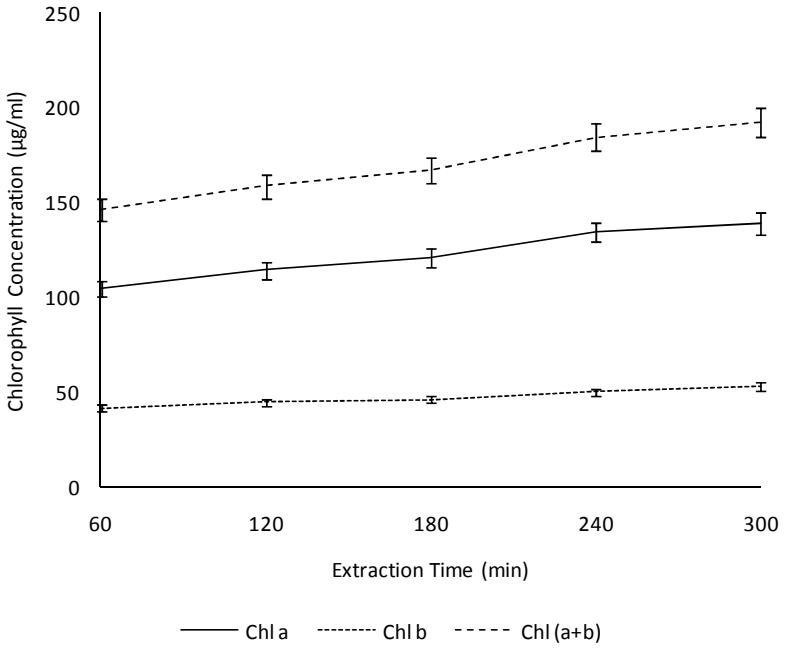

Figure 4-7 - Variation of chlorophyll concentration with time at 40°C

The chlorphyllase activity may be effective around the temperature of 40°C. Hence the initialization of the 1st step of degradation pathway (Section 2.5) would have been started i.e. chlorophyll *a* will break down to form chlorophyllide *a*. Similarly, chlorophyll *b* will be converted to chlorophyllide *b*.

Chlorophyllide molecules are small and more polar compared to chlorophyll molecules. This may facilitate the chlorophyllide molecules to diffuse easily to the solvent. As a result when degradation starts, the concentration of chlorophyllide molecules may increase. Since, chlorophyllide also gives similar peaks at the corresponding wavelengths used in the spectrophotometer; (Barrett *et al.* 1964) the results may indicate a higher yield for chlorophyll at this stage. The increasing trend observed in Figure 4-7, may be due to this reason.

4.2.1.3 The Saturated Condition with the Degradation – at the Temperatures of 50 and 60°C

The concentration of chlorophyll *a*, *b* and *a* plus *b* at the temperatures of 50 and 60°C are shown in Figure 4-8.

Around these temperatures the chlorophyllide may break down further to pheophorbide *a*. Unlike chlorophyllide, pheophorbide give different peak values with chlorophyll in the spectrophotometry, hence the absorption values get reduced. At the

same time, the extraction of chlorophyll has become limited. Hence, at the temperature of 50°C, the concentration of chlorophyll decreases with time as observed in Figure 4-8.

At 60°C the degradation has become prominent than the extraction, after 60 minutes of extraction. This may be due to the starting of the second step of the breakdown of chlorophyll. Hence the reduction of chlorophyll is readily observed. Further mathematical explanation is stated at section 4.8.

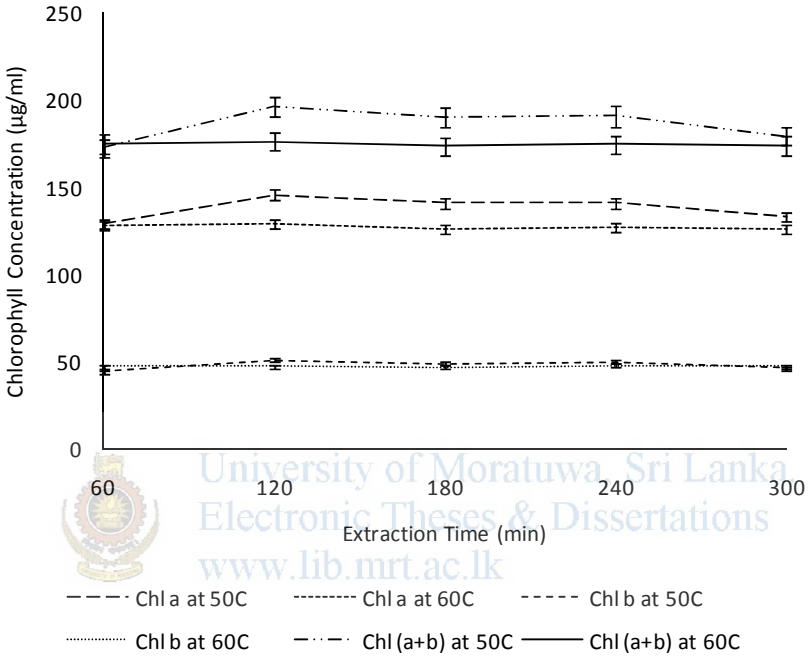

Figure 4-8 – Variation of chlorophyll concentration with time at 50 and 60°C

4.3 Determination of Optimum Time of the Extraction

The concentrations of chlorophyll *a*, *b* and *a* plus *b* obtained for various extraction times at the temperature of 50°C is shown in Figure 4-9.

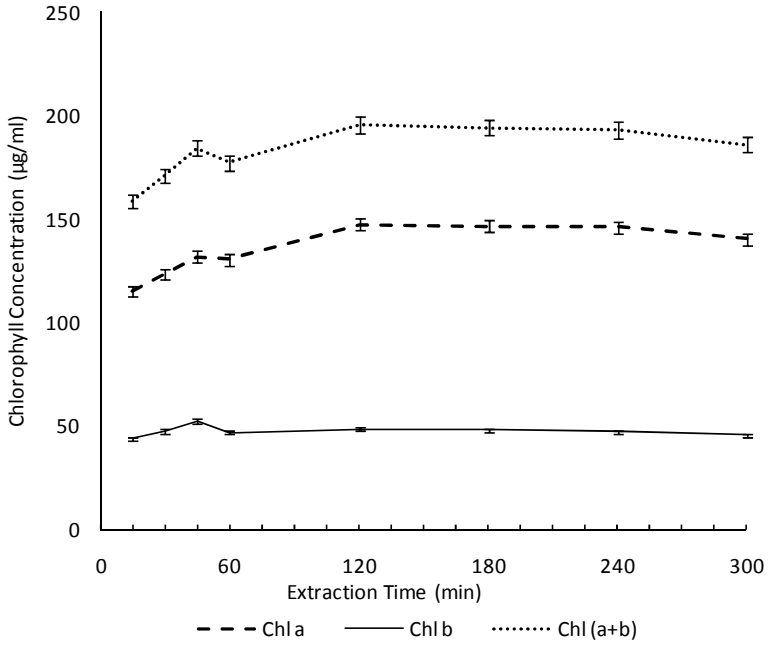

Figure 4-9 – Effect of extraction time on chlorophyll concentration at 50°C

Considering the economical facts, the optimum extraction time was selected as 45 minutes.

4.4 Effective Method of Pre Processing of *A. sessilis*

The weight of extracted chlorophyll *a*, *b* and *a* plus *b* obtained from different pre-processing methods as described in section 3.2.4, were plotted as shown in Figure 4-10. The weight is presented in milligrams per 1g of *A. sessilis* in dry basis.

The highest yield of chlorophyll was obtained from grinding (method 01). Due to the grinding the plant cell wall was disintegrated, as a result the rate of diffusion of the chlorophyll from the cell to the solvent has increased. Blanching had less effect on the rupture of cells than grinding, hence the extraction of chlorophyll is less in method 02 than in method 01. Furthermore blanching had cause to degrade the chlorophyll. Although the weight of chlorophyll gained from method 03 (grinding after blanching) is higher than the method 02, it is less than that in method 01. It shows that grinding has improved the extraction of chlorophyll and blanching has reduced the amount of chlorophyll extracted. In expelling (method 04), the samples were not exposed to any degradation, yet the chlorophyll is less than that in method 01, because the expelling has done less disintegration of cell wall than the mechanical grinding in method 01. The

degradation of chlorophyll is less in drying (method 05) than in method 02, because of the low temperature used in the heat treatment.

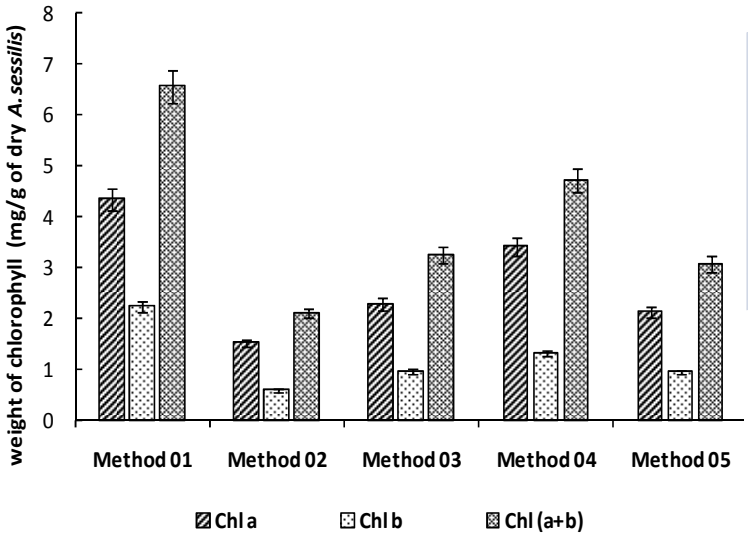

Figure 4-10 - Weight of chlorophyll a, b and a plus b for different pre-processing methods

The results clearly show that the mechanical methods of disintegration of cell walls are superior to the other methods. Therefore the mechanical grinding described in section 3.2.4.1 is selected as the optimum pre-processing method for further studies.

The weight of the total chlorophyll that was obtained by grinding as the pre-processing method was 6.543 mg/g of *A. sessilis* (dry basis). Meanwhile chlorophyll *a* and *b* weights were 4.327 and 2.215 mg/g of *A. sessilis* (dry basis) respectively.

4.5 Determination of the Effective Storage Condition for *A. sessilis*

The weight of extracted chlorophyll *a* and *b* obtained from varying the storage conditions as described in section 3.2.5, were plotted as shown in Figure 4-11 and Figure 4-12, respectively. The weight is presented in milligrams per 1g of *A. sessilis* in dry basis.

Although the reduced amount of chlorophyll *a* is comparatively low in the samples stored at environment 01 and 02, the rest of the samples have shown a considerable reduction of chlorophyll *a*. Chlorophyll *b* has followed the same trend.

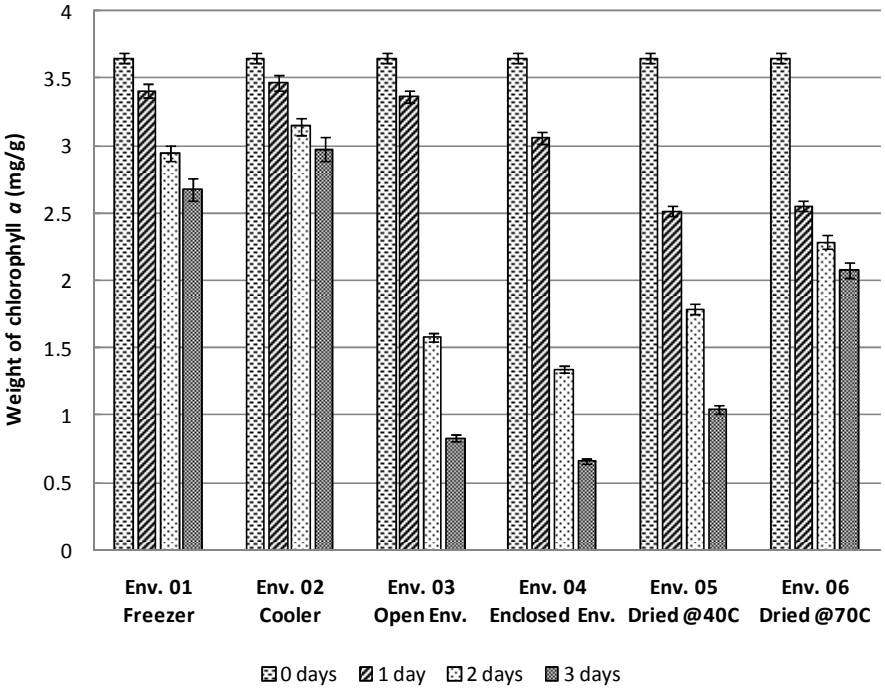

Figure 4-11 - Effect of storage condition on yield of chlorophyll a

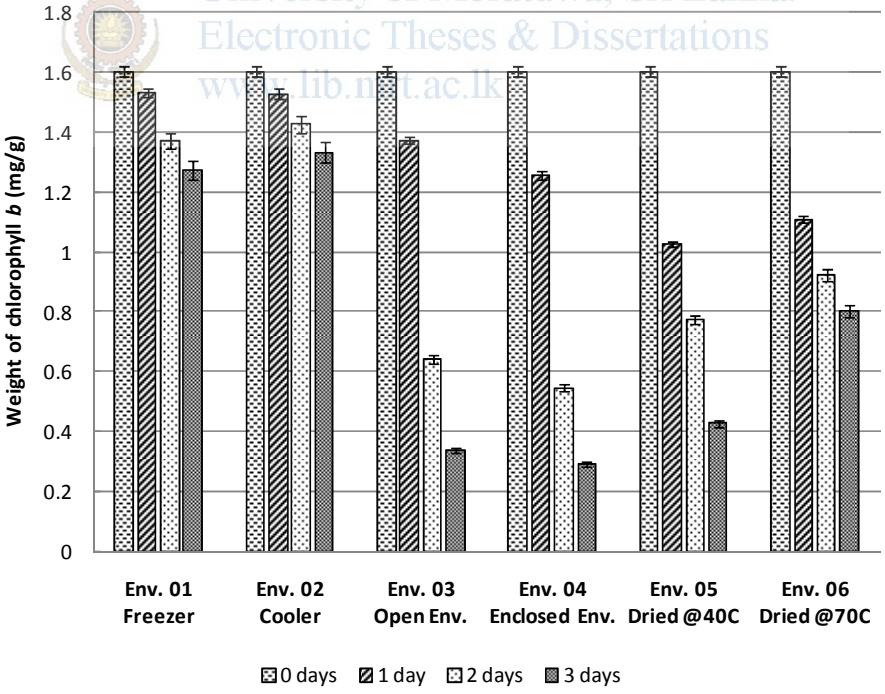

Figure 4-12 - Effect of storage condition on yield of chlorophyll b

The percentage loss of chlorophyll *a* and *b* for each day, is calculated using the data shown in Figure 4-11and Figure 4-12, and is shown in Figures 4-13, 4-14 and 4-15.

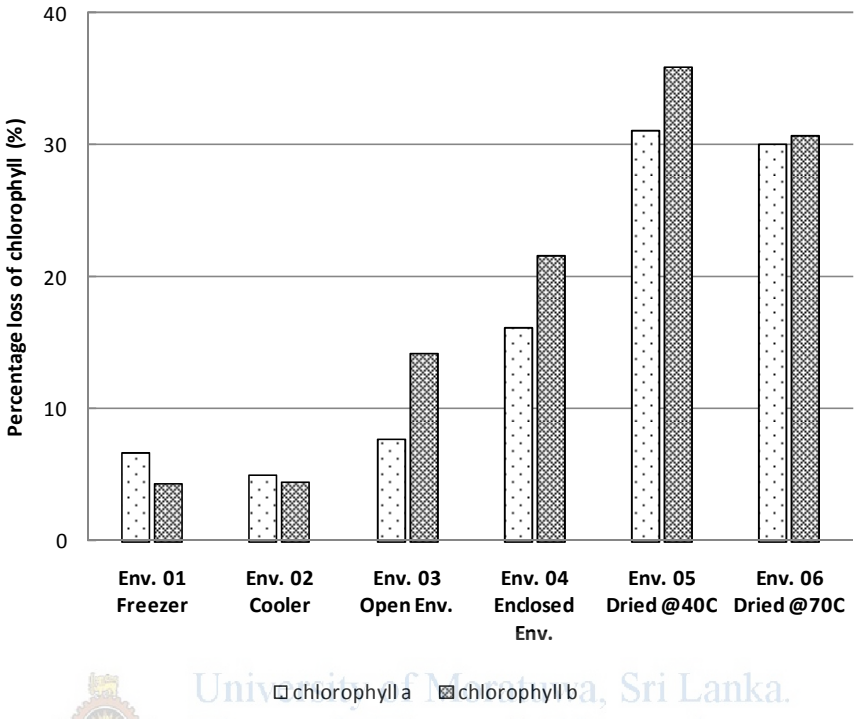

Figure 4-13 – The loss of chlorophyll after 1 day of storage

The percentage losses of chlorophyll *b* after storage in all the environments except in environment 01 and 02 are higher than the loss of chlorophyll *a* (Figure 4-14 to 4-15). Although the rate of degradation of chlorophyll *b* is relatively low compared to that of chlorophyll *a*, loss of chlorophyll *b* can be higher than chlorophyll *a* during the initial step of the degradation pathway, as described in section 2.5. i.e. In the presence of chlorophyll *b* reductase, chlorophyllide *b* converts into chlorophyllide *a*.

It has shown that adenosine triphosphate (ATP) is required to activate chlorophyll *b* reductase (Ito *et al.* 1994, 1996). Further, ATP content has shown a tendency to decrease at chilling and freezing temperatures (Sobczyk *et al.* 1985). At lower temperatures, ATP content in *A. sessilis* might be reduced slowing the activation of chlorophyll *b* reductase. Hence the degradation of chlorophyll *b* gets delayed depicting a lower rate of reduction than chlorophyll *a*.

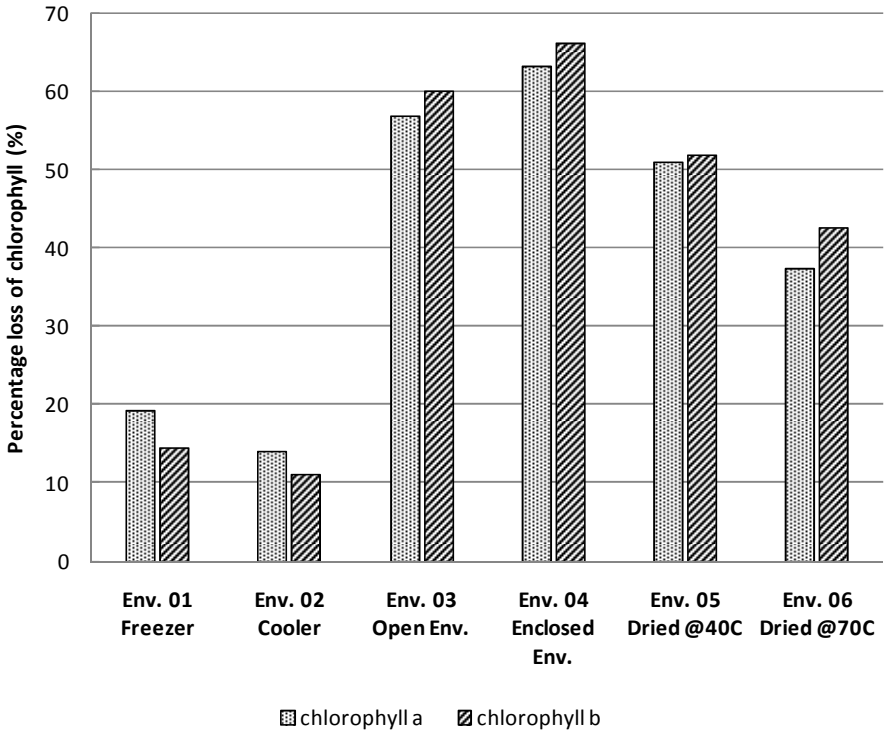

Figure 4-14 - The loss of chlorophyll after 2 days of storage

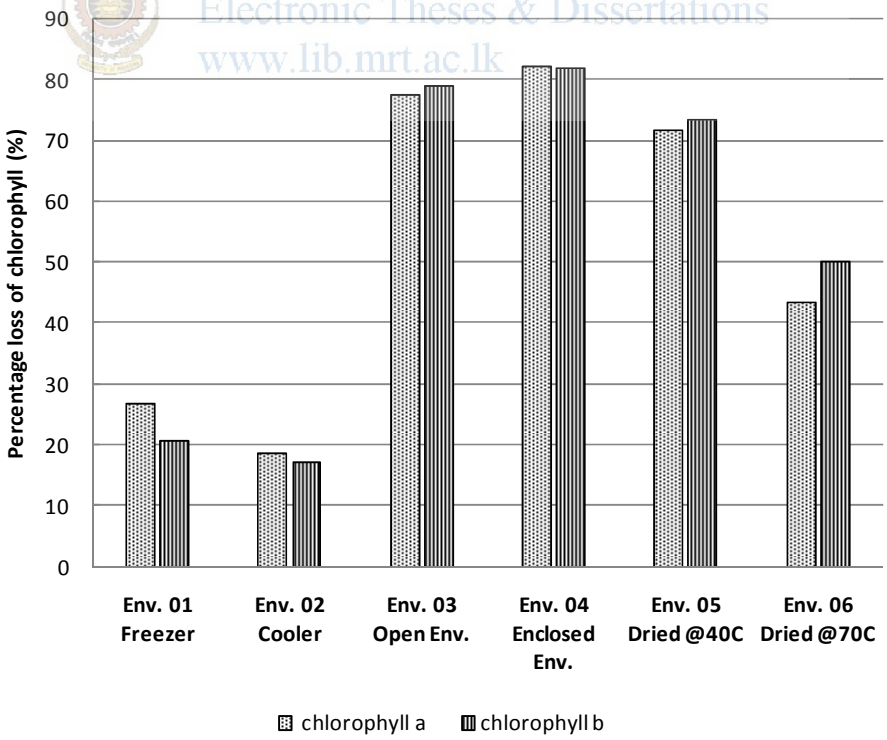

Figure 4-15 - The loss of chlorophyll after 3 days of storage

The percentage loss of chlorophyll *a* plus *b* for each day is shown in Figure 4-16.

Loss of chlorophyll of the samples stored under the conditions of environment 01 and 02 is lower than the other samples. The temperatures of the environment 01 and 02 are lower than the rest, and the low temperatures affect to reduce the effect of degradation of chlorophyll, hence the loss of chlorophyll has reduced. The percentage loss of chlorophyll in environment 01 is higher than the loss in environment 02. The cause may be the chilling injury of *A. sessilis* due to the cold storage at the temperature of 5°C. When comparing the samples of environment 03 and 04, the latter has a higher loss because the sample has subjected to degradation due to limited light and oxygen conditions. The samples of environment 05 have a higher percentage of loss of chlorophyll than of environment 06. This may be due to the increase of rate of degradation during the extraction. Samples of environment 05 were subjected to a temperature of 40°C during the storage and then again subjected to 50°C during the extraction.

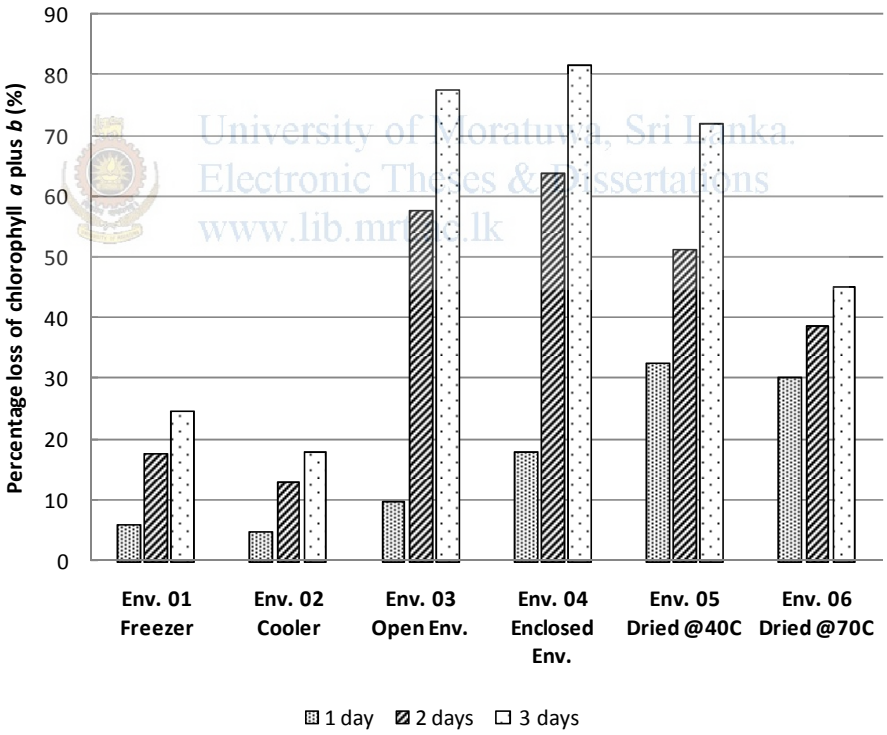

Figure 4-16 - Effect of storage condition on the percentage loss of chlorophyll a plus b

As stated in section 4.2.1.2, the sample contains chlorophyll molecules as well as chlorophyllide before the extraction. Once it is again subjected to a temperature of 50°C the diffused chllorophyllide may also degrade further. But when the samples are dried to

70°C and then extracted at 50°C only the chlorophyll will degrade. Figure 4-11 and Figure 4-12 also supports for this suggestion.

After 3 days of storage the samples stored in environment 02 has shown a 17.9% loss of chlorophyll. The rest of the samples have more than 20% loss of chlorophyll. For 1 day storage, the samples of environment 01, 02 and 03 have shown a loss which is less than 10%. Considering the economical factors, the conditions of environment 03 is selected as the optimum storage conditions for 1 day storage, while the conditions of environment 02 is selected for a longer storage.

4.6 Production of Sodium Copper Chlorophyllin

Although the same reactions have taken place, the methodology used to produce sodium copper chlorophyllin has some modification over the methods of Lee 1989 and Hendry 1996. In this method, the intentional saponification has eliminated, since the solution is already saponified during the extraction, because of the buffer solution that has used. Acidification with copper salt is also replaced by the heat treatment with copper metal since it reduces the involvement of chemicals. Instead of isolation of sodium copper chlorophyllin by fractionation and washing, the whole extract is used with the other pigments such as carotene and xanthophylls.

The conversion of chlorophyll and its derivatives is 33.3%.

4.7 Study of the Kinetics of Degradation of Chlorophyll

When a reaction is known to follow a first order kinetic model, the concentration of the reacting substance is related to time, at a constant temperature as follows,

$$\ln\frac{C}{C_0} = -kt \qquad 4\text{-}1$$

Where,

C - The concentration at any time

C_0 - The concentration at time zero

k - Rate constant at the reaction temperature

t - Time

The normalized concentration of chlorophyll *a* and *b* at each temperature are shown in Figure 4-17 to 4-20. Since the plots of the logarithm of the normalized concentration $\left(C/C_0\right)$ against time, is compared to a straight line with a high validity (with a minimum R^2 of 0.9991), a first order kinetic relationship is identified.

The rate constant values obtained are shown in Table 4-1 and the increase of k with temperature can be readily observed.

Table 4-1 – The rate constants at various temperatures

	Temperature (°C)	k
Chlorophyll *a*	15	0.0002296
	30	0.0003053
	40	0.0003506
	50	0.0004079
Chlorophyll *b*	15	0.0002175
	30	0.0002777
	40	0.0003278
	50	0.0003669

Considering the relationship between the rate constant and the temperature, the Arrhenius equation of the following form can be used to relate the two parameters.

$$\ln k = \ln A_0 - \left(\frac{E_0}{R}\right)\frac{1}{T} \qquad 4\text{-}2$$

Where,

k - Rate constant

A_0 - Pre-exponential constant

E_0 - Activation energy

R - Gas constant temperature in Kelvin

The plot of the logarithm of rate constant against 1/T is compared with the Arrhenius plot and it is shown in Figure 4-21. Since the validity of the model is high (minimum R^2 is 0.9985) and therefore the comparison is agreeable, the values were calculated for activation energy and for A_0 and are shown in Table 4-2.

Table 4-2 – The activation energy of chlorophyll and A_0

	Activation energy E_0 (kcal/mol)	Pre-exponential constant A_0
Chlorophyll *a*	3.0143	0.0449
Chlorophyll *b*	2.78	0.0282

Although the values obtained here are slightly lower when compared to the degradation kinetics of natural chlorophyll *a* and *b* which is 17.5 and 17kcal/mol (Gupte *et al.* 1964) and 143 and 23kcal/mol (Ryan-Stoneham *et al.* 2000) respectively. But the values are in good agreement with the studies done on chlorophyll derivatives, which is 2.80 to 14.0 kcal/mol for chlorophyll *a* and 1.84 to 11.0 kcal/mol for chlorophyll *b* (Koca *et al.* 2007).

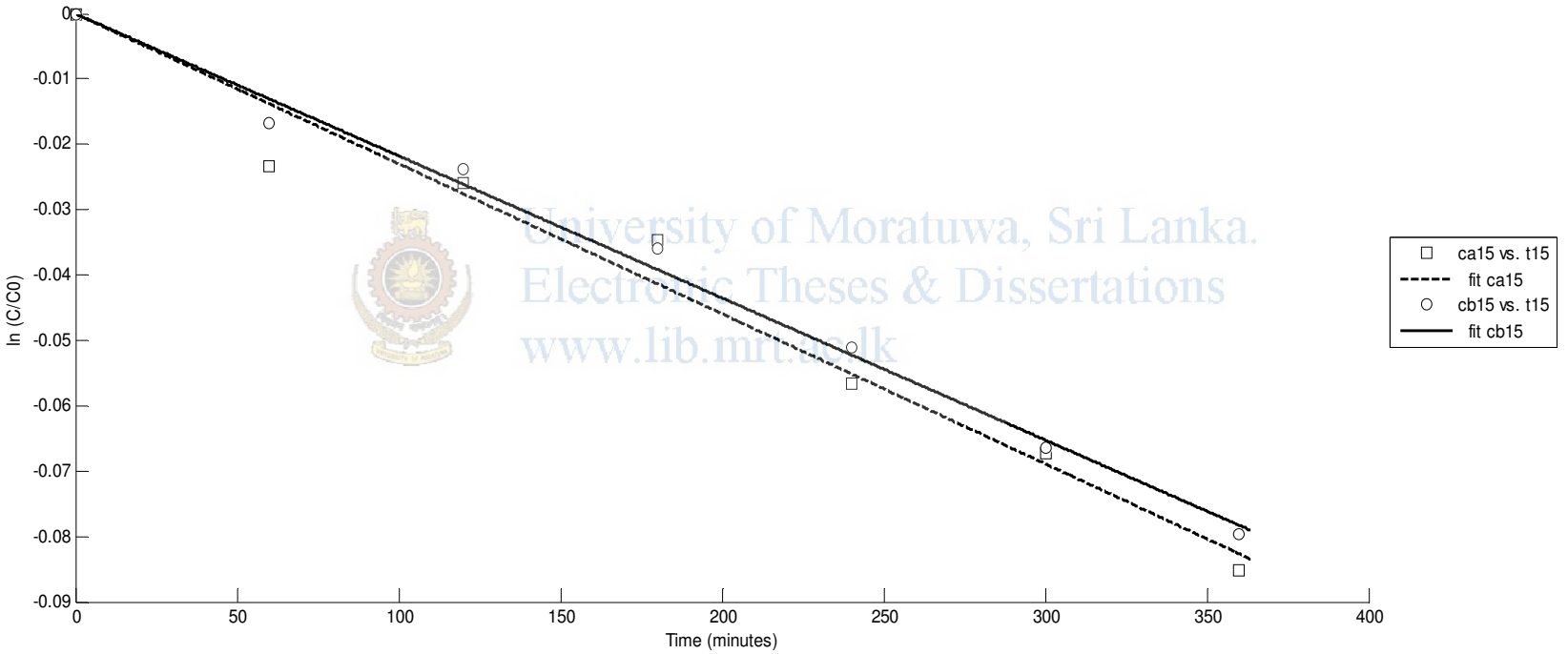

Figure 4-17 – The relationship between the concentration of chlorophyll and time at 15^{o}C

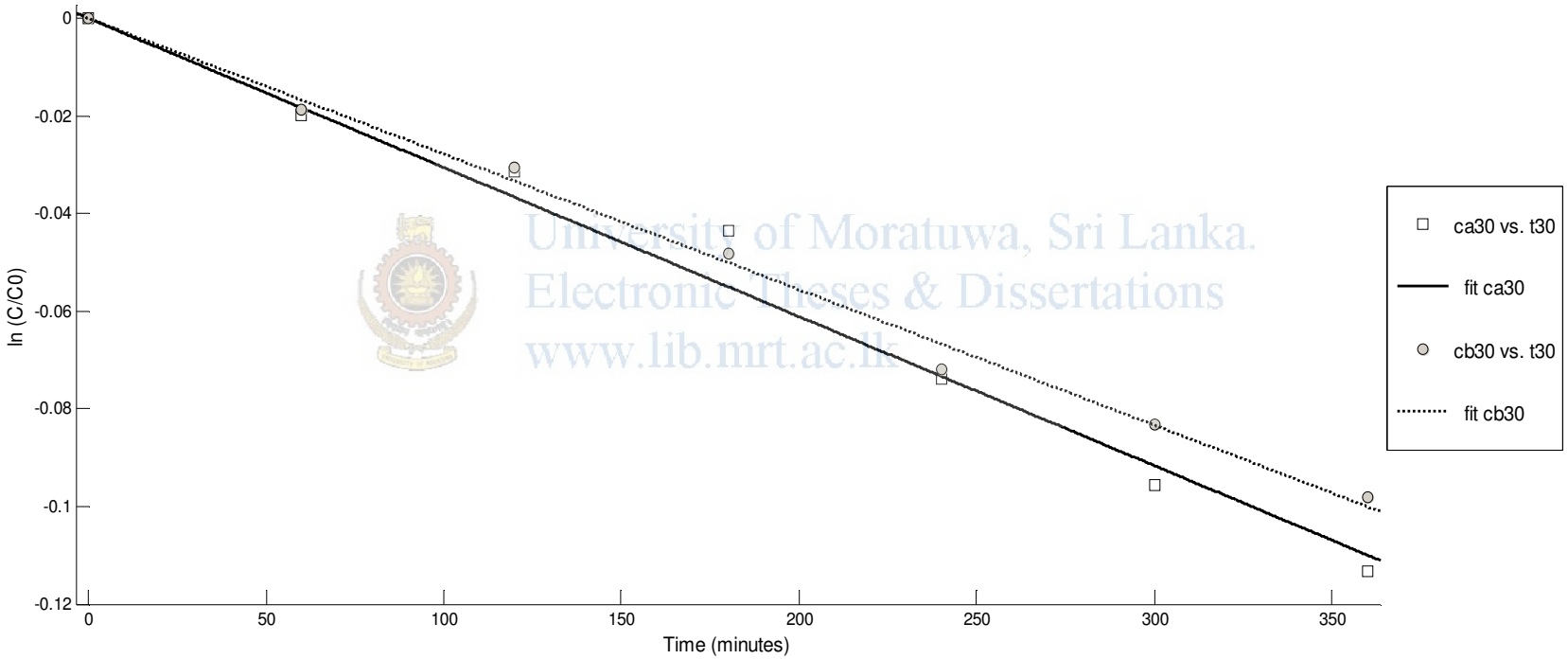

Figure 4-18 - The relationship between the concentration of chlorophyll and time at 30°C

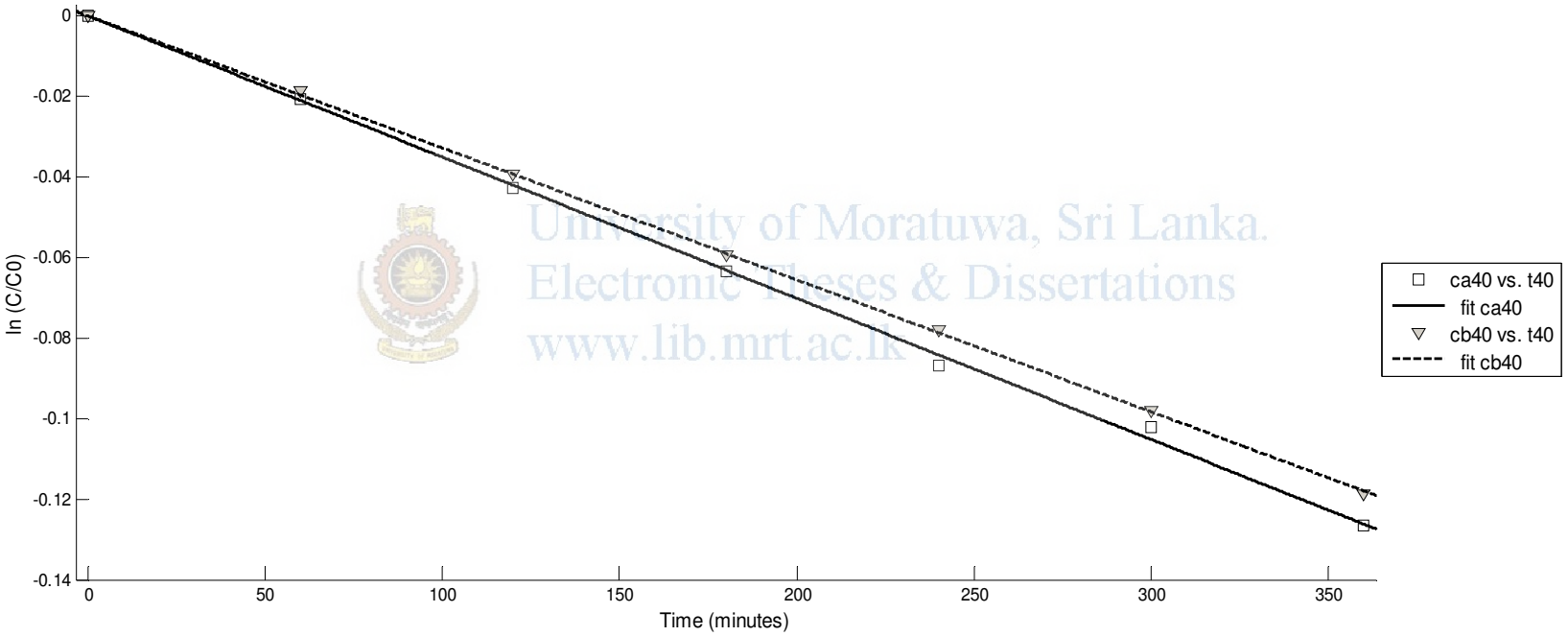

Figure 4-19 - The relationship between the concentration of chlorophyll and time at 40°C

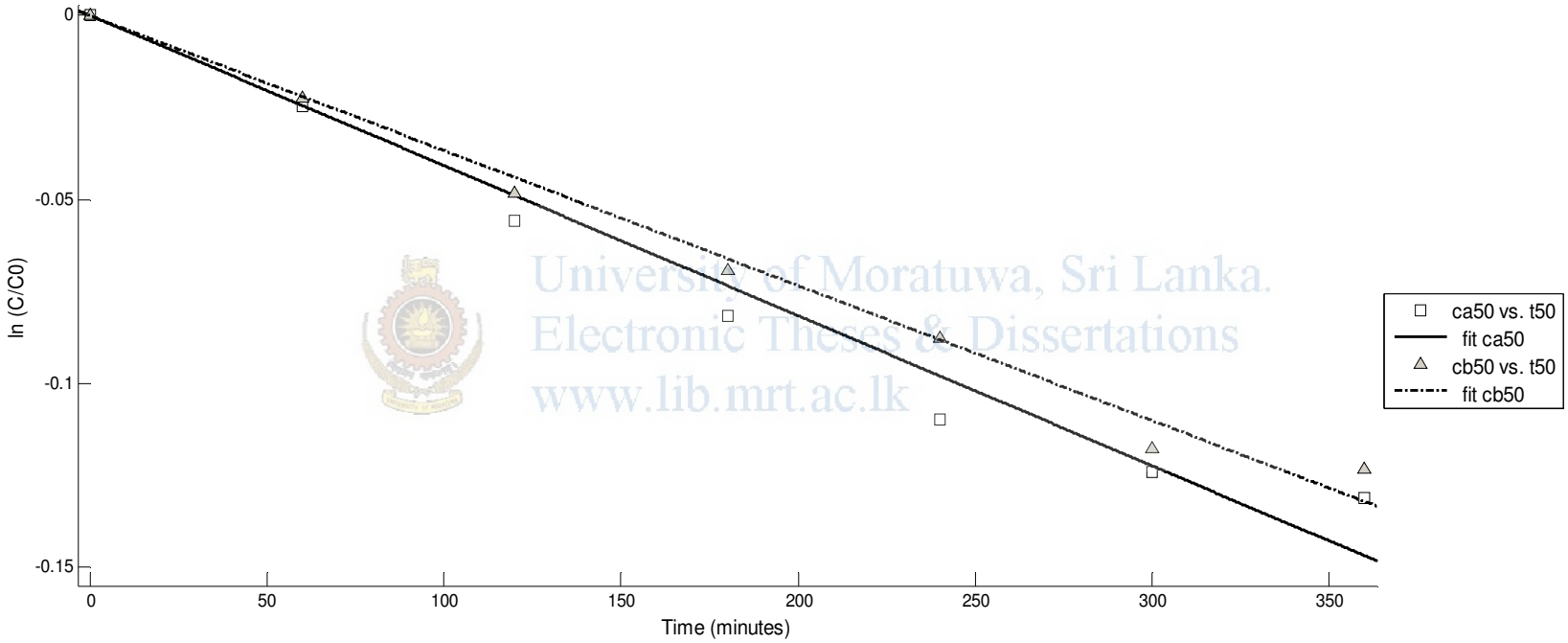

Figure 4-20 - The relationship between the concentration of chlorophyll and time at 50°C

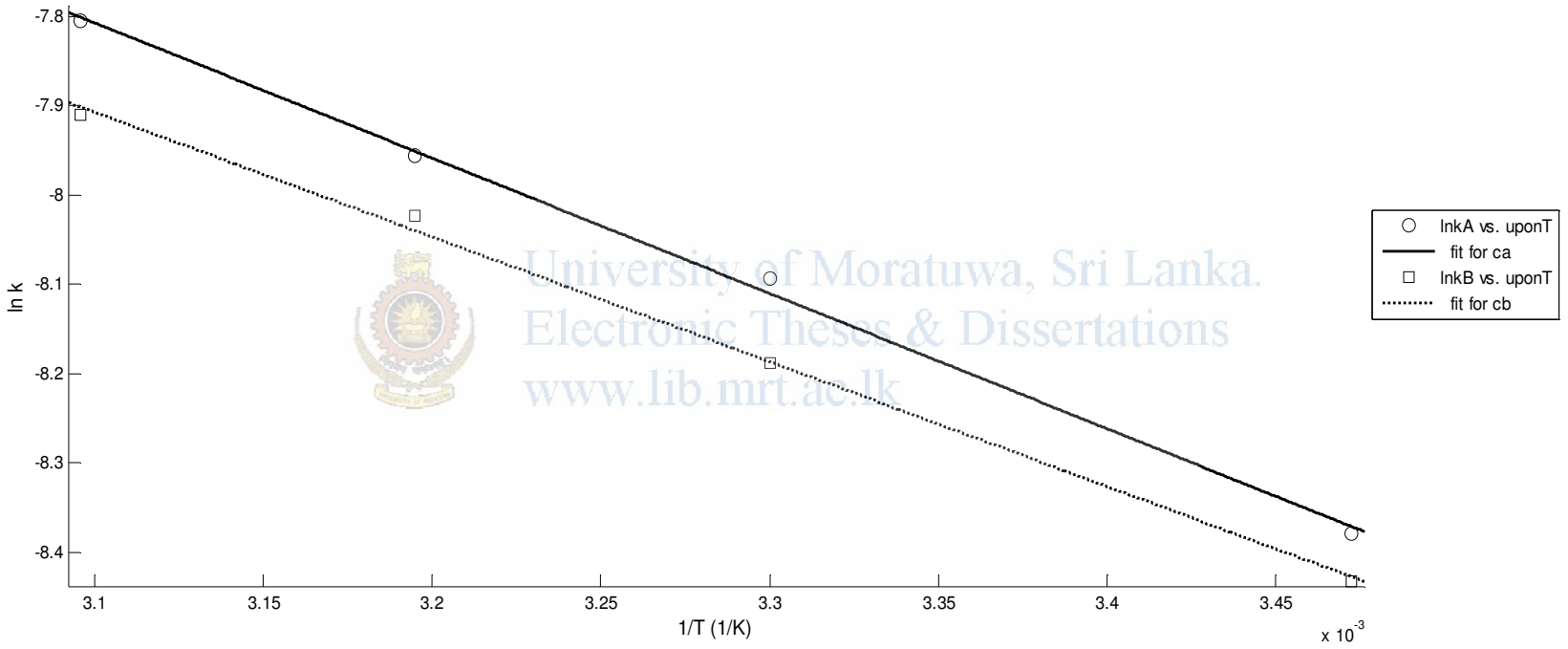

Figure 4-21 – The relationship between the rate constant and the temperature

4.8 Comparison of the Mass Transfer with a General Mass Transfer Model and the Estimation of Saturation Solubility of Chlorophyll *a* and *b* in 80% (v/v) Acetone

4.8.1 Approximation of the Mathematical Model to the Experimental Values

The experimental results shown in Figure 4-3 and Figure 4-4, were smoothed with Loess (quadratic) method using the program; Matlab™ 7.5.0. The smoothed concentrations of chlorophyll *a* and *b* were plotted against the residence time.

The observational data modeled by the equation *3-6*, were also plotted in the same graph. Both the theoretical and experimental results, obtained for the concentration of chlorophyll *a* and *b* are shown in Figure 4-22 and Figure 4-23, respectively.

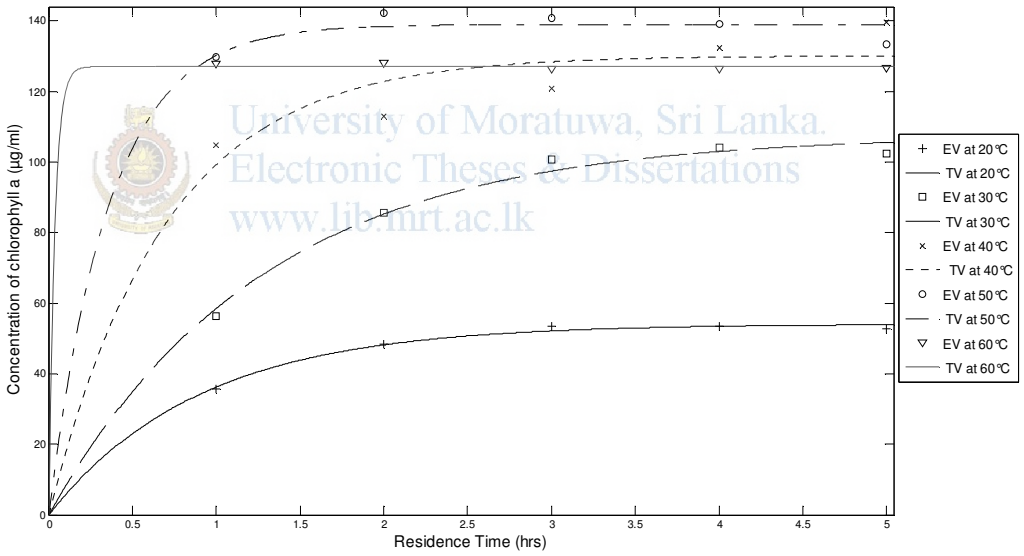

Figure 4-22 – Approximation of theoretical values on concentration of chlorophyll a (EV and TV stand for Experimental and Theoretical Values respectively.)

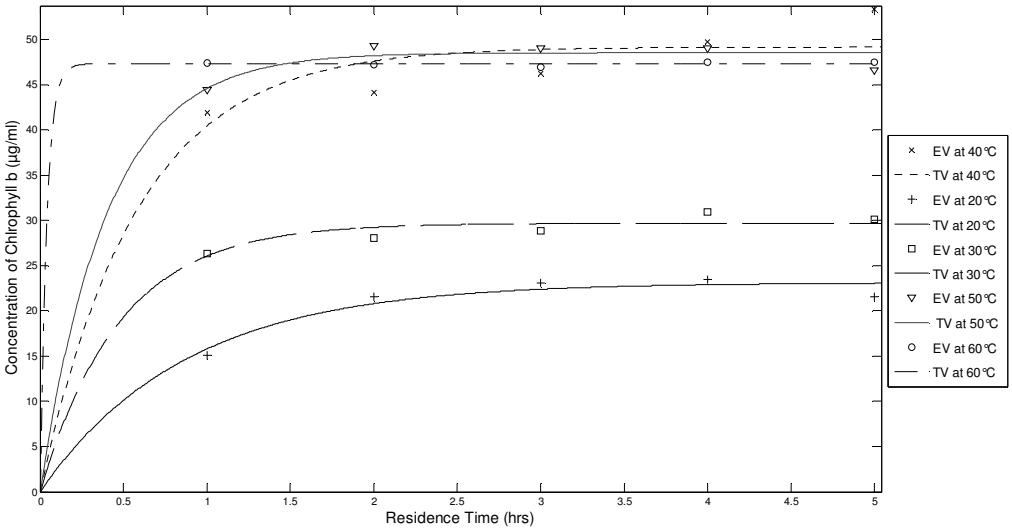

Figure 4-23 – Approximation of theoretical values on concentration of chlorophyll b (EV and TV stand for Experimental and Theoretical Values respectively.)

4.8.2 Validation of the Model

The validation was done according to the three methods described in section 2.7.

4.8.2.1 Residual Analysis

The residuals for the plots are shown in Figure 4-24 and Figure 4-25.

Although the residuals appear randomly scattered around zero, indicating that the model describes the data well, it cannot be determined only with the residual analysis because the number of data points per data series is low. i.e. only 5 data points.

4.8.2.2 Confidence and Prediction Bounds

The confidence level for the bounds is used as 95%.

The prediction bounds for the data of chlorophyll *a* and *b* are shown in Figure 4-26 and Figure 4-27 respectively.

As shown in Figure 4-26 and Figure 4-27, very wide interval for the fitted coefficients at 40˚C indicates that the model does not fit well. But for the other temperatures, it cannot be concluded whether the model fits the data or not, by using only this method. Therefore the GoF statistics are used.

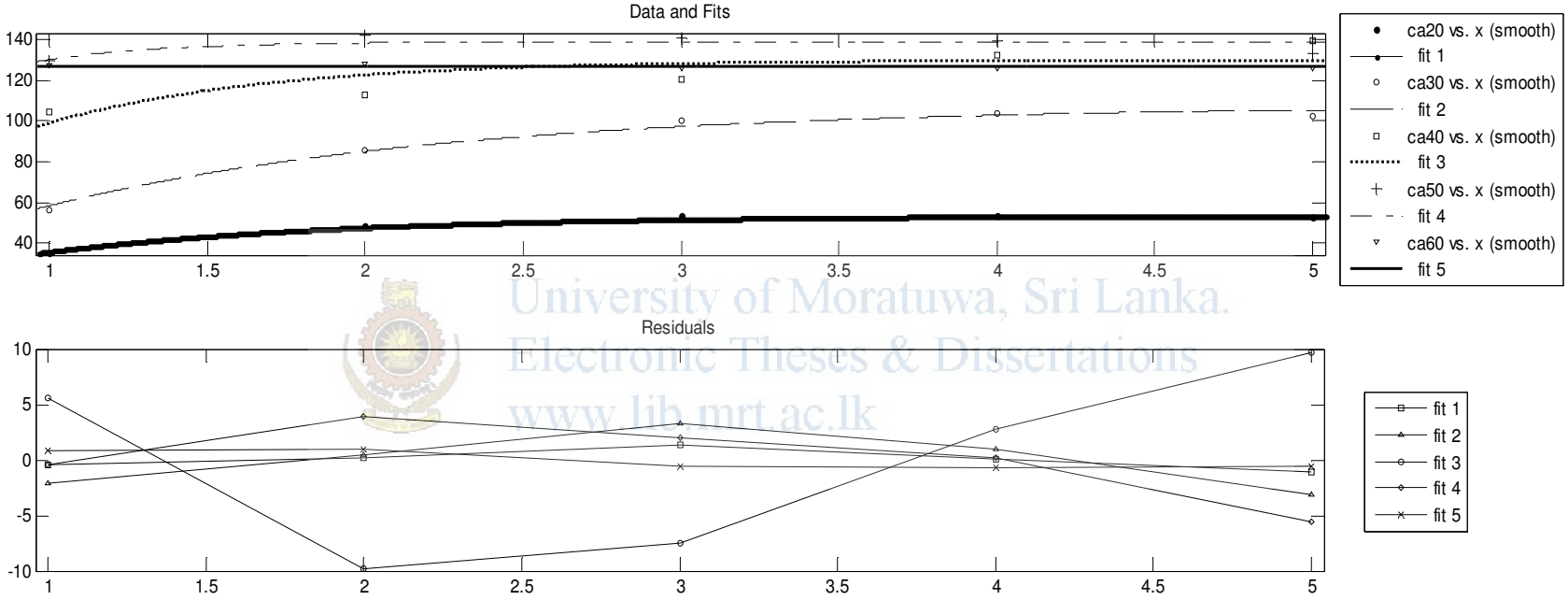

Figure 4-24 – residuals for concentration of chlorophyll a

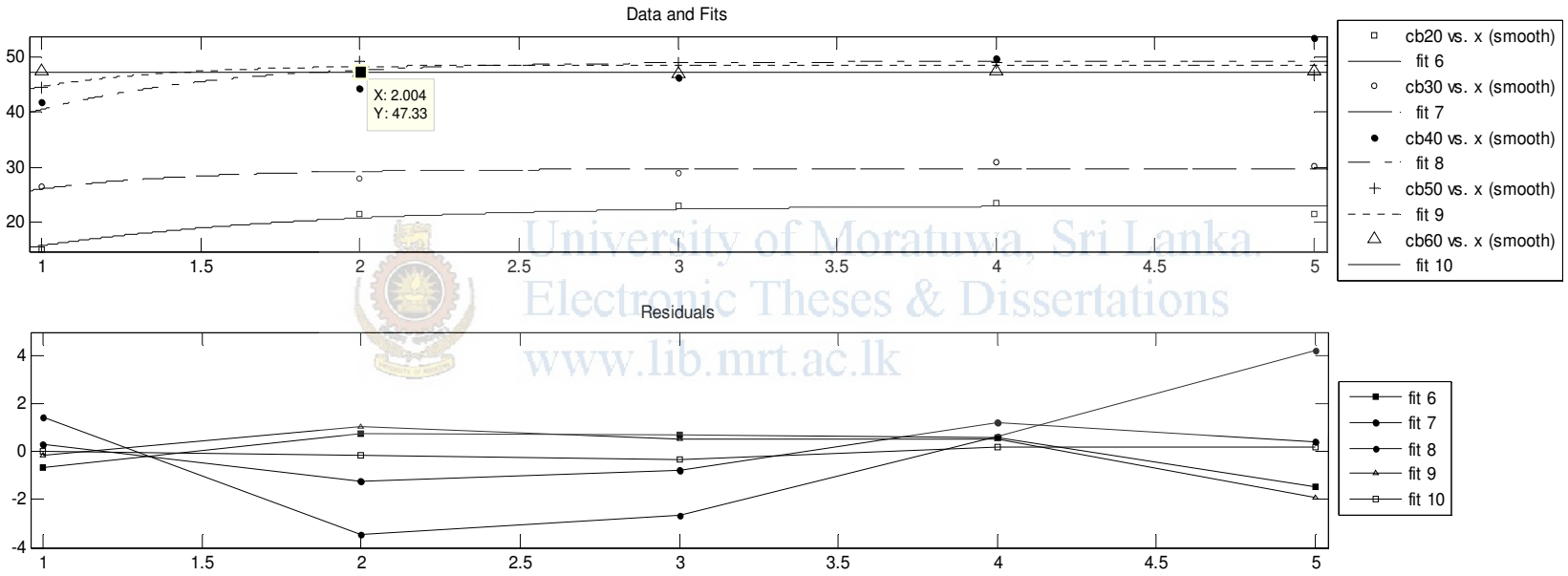

Figure 4-25 - residuals for concentration of chlorophyll b

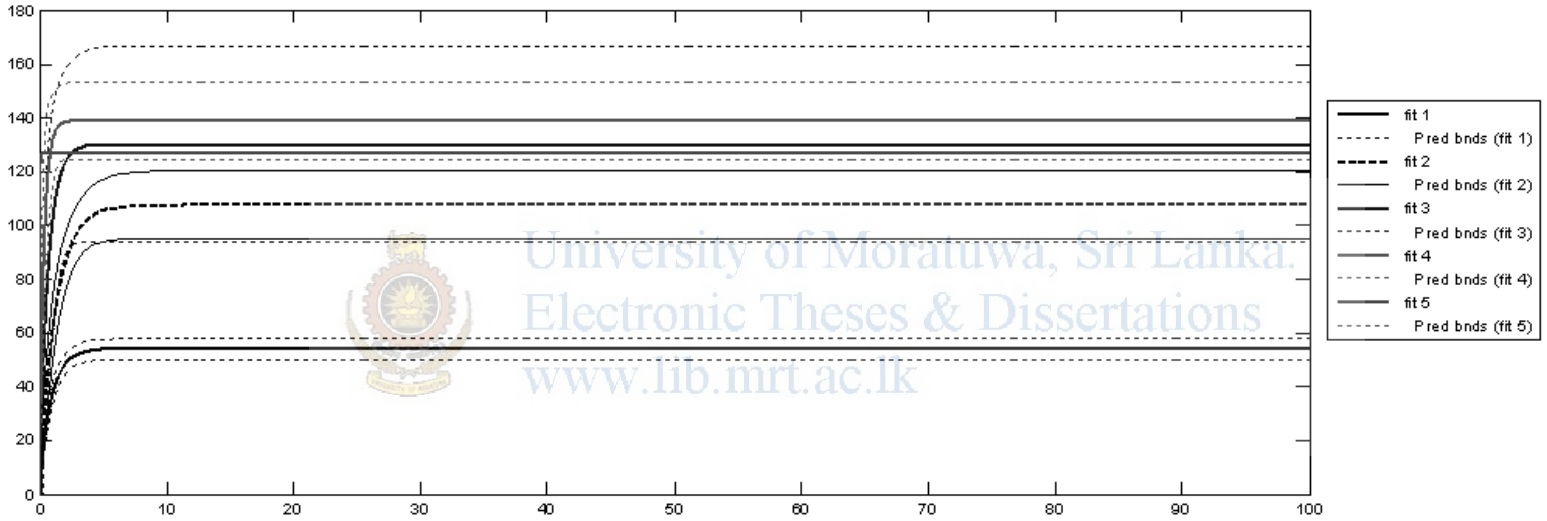

Figure 4-26 – prediction bounds for the model for the extraction of chlorophyll a

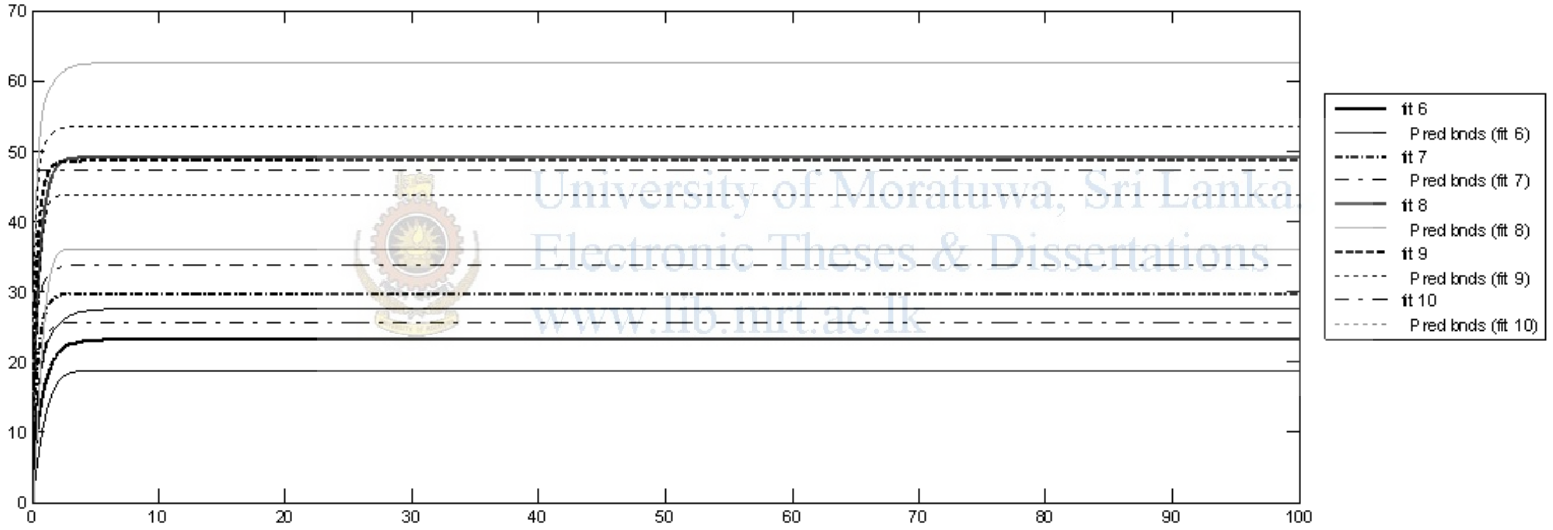

Figure 4-27 - prediction bounds for the model for the extraction of chlorophyll b

4.8.2.3 GoF Statistics

The obtained values for the coefficients described in section 2.7.3, are shown in Table 4-3.

Table 4-3 - Correlations of actual and predicted data

Temperature	Type of Chlorophyll	SSE	R^2	Adjusted R^2	RMSE
20°C	*a*	3.322	0.9856	.9807	1.052
	b	3.974	0.9133	.884	1.151
30°C	*a*	2.648	0.9836	.9781	2.971
	b	3.859	0.6945	.693	1.134
40°C	*a*	261.6	0.6415	.522	9.766
	b	39.32	0.5256	.3675	3.62
50°C	*a*	49.81	0.5635	.418	4.075
	b	5.336	0.7059	.6079	1.334
60°C	*a*	2.796	≈0	Minus value	0.9654
	b	0.2055	≈0	Minus value	0.2617

The coefficients for the temperature of 20 and 30°C for chlorophyll *a,* indicate the best correlations of actual and theoretical data explaining over 97% variation of the observed data. For the temperature of 20°C for chlorophyll *b*, the model shows a good correlation with the experimental data with the explanation of more than 88% variation. The model for the temperature of 30°C for chlorophyll *b* shows an acceptable approximation for experimental data, with explaining more than 69% variation itself.

The rest of the data showed in Table 4-3, cannot be considered as reasonable approximation to the experimental data. The predicted and the actual data are significantly different. This result suggests that the mathematical model for mass transfer does not fit well for the extraction at temperatures 40, 50 and 60°C. The cause was suggested as the degradation of chlorophyll. Hence this approximation supports the hypothesis described in section 4.2.1.

4.8.3 Conclusion of the Validation and Determination of the Value for C_{AS} and K .

The extraction of chlorophyll *a* and *b* at the extraction temperatures of 20 and 30°C can be expressed by *Equation 3-6*. Hence it is in accordance with the general mass transfer behavior.

The values obtained for C_{AS} and K are shown below.

Table 4-4 - values for C_{AS} and K

Type of Chlorophyll	Temperature ˚C	C_{AS}	K (×10^{-4})
a	20	54.06	3.075
	30	107.6	4.954
b	20	23.13	3.189
	30	29.68	5.842

Only the values for the temperature of extraction at 20 and 30°C are shown.

The saturation solubility of chlorophyll *a* at the temperature of 20 and 30°C were 54.06 and 107.6 µg/ml respectively and for chlorophyll *b* were 23.13 and 29.68 µg/ml. With the increase of temperature, the increase of K values indicates the general characteristic of leaching behavior for most of the solid-liquid extraction processes.

5 Conclusion and Recommendations

This chapter includes the conclusion of the work done and the recommendations for future work.

5.1 Conclusions

Optimum operating conditions of extraction of chlorophyll from *A. sessilis* using 80% acetone was studied. The optimum operating conditions for the parameters considered are listed below.

The solvent volume to *A. sessilis* weight ratio	5:1
Temperature of extraction	50°C
Time of extraction	45 minutes

Post harvesting operations for *A. sessilis* was also studied and the two conditions that were considered are listed below.

Method of pre processing: Grinding – mechanical (5minutes)

Condition of storage: 1 day storage- ambient air condition (indoor)

Storage of more than 1 day - refrigerator (15°C)

The results obtained for extraction of chlorophyll at the temperatures of 20 and 30°C were fitted to the general mass transfer models. However for high temperature extractions at 40, 50 and 60°C, results cannot be fitted into the general mass transfer models due to the degradation of chlorophyll. The saturation solubility of chlorophyll *a* was 54.06 and 107.6µg/ml and that for chlorophyll *b* it was 23.13 and 29.68 µg/ml at 20 and 30°C respectively.

Sodium copper chlorophyllin has produced with a 33.3% conversion of chlorophyll and its derivatives, according to a modified process.

The kinetics of degradation of chlorophyll of the produced sodium copper chlorophyllin follows a first order relationship between the concentration and the time. The relationship between the rate constant and the temperature follows the Arrhenius behavior.

The activation energy for chlorophyll *a* and *b* were 3.0143 and 2.78 kcal/mol, respectively. The rate constants obtained for chlorophyll *a* at different temperatures are higher than that of chlorophyll *b*.

5.2 Recommendations

1. The dual effect of degradation and extraction on the yield of chlorophyll stated in section 4.2.1 can be verified using more analytical quantification methods like HPLC.

2. The effect of aging and the effect of varieties of *A. sessilis* on extraction of chlorophyll should be studied.

3. The pilot scale of the process of production of sodium copper chlorophyllin should be studied.

6 Reference

This chapter includes the list of the literature that was cited throughout the work.

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Appendix

The scientific classification of *A. sessilis* is as follows.

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Caryophyllales
Family:	Amaranthaceae
Subfamily:	Gomphrenoideae
Genus:	Alternanthera
Species:	*sessilis*

Synonyms: *Alternanthera triandra* Lam., *Altemanthera denticulate* R. Br., *Altemanthera nodiflora* R. Br., *Altemcinthera repens* Gmel

Vernacular Name: Sinhala: Mukunuwenna; Tamil: Ponnannkannjkkirai; English: dwarf copperleaf, joy weed

Description: A much branched prostrate herb, branches often purplish, frequently rooting at the lower nodes; leaves simple, opposite, somewhat fleshy, lanceolate, oblanceolate or linera-oblong, obtuse or sub-acute, sometimes obscurely denticulate, glabrous, shortly petiolate; flowers small, white, in axillaiy clusters; fruits compressed obcordate utricles, seeds suborbicular.

Propagation: By seeds

Part Used: Whole plant.

Chemical Constituents: -Sitosterol, stigmasterol, campesterol, cc-spinasterol, oleanolic acid rhamnoside, 24-methylene cycloartenol, cycloeucalenol, lupeol, 5-cc-stigmasta-7-enol and its palmitate.

Uses: The plant is bitter, sweet, astringent, acrid, cooling constipating, depurative; digestive, cholagogue, galactagogue and febrifuge and is useful in vitiated conditions of kapha and pitta, buring sensation, diarrhoea, leprosy, skin disease, dyspepsia, haemorrhoids agalactia, splenomegaly and fever.