CHAPTER III

EXPERIMENTAL

Materials

The following materials were obtained from commercial sources.

Drugs and Excipients

- Glibenclamide BP (Batch No.GC/002/4063, Cadila Healthcare Limited, India)
- Acetylsalicylic acid working standard 100.05% (Lot No.0505203, Rhodia Thai Industries Ltd., Thailand)
- Acetylsalicylic acid Powder (Lot.0600602, Rhodia Thai Industries Ltd., Thailand)
- Aspirin tablet 325 mg (L/C No. I10429, British Dispensary, Thailand)
- Salicylic acid (Lot.0435400, Rhodia Thai Industries Ltd., Thailand)
- Poly-n-isopropylacrylamide Cross linked (Batch No. RE-69887, Carbomer, Inc., USA)
- Red Cabbage Extract Powder 30 WSP (Chr.Hansen Company, Denmark. Distributed by The East Asiatic (Thailand)
- Hydroxypropyl methylcellulose (MethocelE15LV® Batch No.RD14012406 Colorcon, Inc., UK) Distributed by Rama Production Co., Ltd.
- Ethyl cellulose (Ethocel® 10 cps., Lot.20013T01, Colorcon,Inc.,UK)

- Sodium Alginate (Lot No.105035/2001 BL669, Germany)
- Calcium chloride dehydrate (Batch No. AF703220, Ajax Finechem, New Zealand)
- Potassium bicarbonate (Lot. No.A673954, Merck, Germany)
- Orthophosphoric acid (Batch No.A3B017, Ajax finechem, New Zealand)
- Acetic acid Glacial (Lot K32754317 349, VWR International., Ltd., England)
- HPLC Grade Acetonitrile (Burdick & Jackson, Korea)
- HPLC Grade Methanol (Burdick & Jackson, Korea)
- Polyethylene glycol 400 USP/NF (Lutrol®E400 Lot.82-1765 BASF, Germany)
- Dibutyl phthalate (Lot 61219343, MERCK-Schuchardt, Germany)
- Potassium Dihydrogen Orthophosphate (Batch No.F2H145, Asia Pacific Specialty Chemicals Ltd., Australia)

Equipment

- Differential Scanning Calorimeter (DSC) (Mettler Toledo, Model DSC822e, Switzerland)
- X – ray powder diffractometer (Bruker AXS Model D8 Discover, Germany)
- Image Scanner (Lexmark X83, United States)
- Adobe PhotoShop® Software (Photoshop® version 7.0)
- Hot air oven (Model 100, Memmert, Germany)
- Shaker bath (Model 28L/8/SH/C, PolyScience Co., Ltd., USA)
- High performance liquid chromatography (Model LC-20AB, Zhimadzu, Japan)
- Pressure compressed tableting machine (model 4350L, Carver, Inc, USA)
- Stirrer (Janke & Kunkel Kika – Labor technik. RW 10R)
- pH meter (model210A+, Thermo Orion, Germany)
- Analytical Balance (Model A200S, Sartorius, Germany)
- Analytical Balance (Model SK-20KWP, A&D Co., Ltd., Korea)
- Spray gun for Thai Coater®15" equipment (Pharmaceutical and Medical supply, Ltd., Thailand)

Methods

The experiment was performed in four parts. The first two parts were to investigate the different modes of degradation and degradation kinetics of the model drugs to glibenclamide and aspirin to formulate the proper compositions of the films as drug degradation indicator. The last two parts were to develop thin films appropriate for the rate and mechanisms of degradation for both model drugs.

1. Glibenclamide

Effect of storage temperature and time on glibenclamide degradation has been shown to be due to solid state transformation. Solid state techniques were used to characterize solid forms such as X-ray powder diffractometry (XRD) and differential scanning calorimetry (DSC). The solubility studies were also performed to confirm the critical change after solid state transformation.

1.1 Preparation of Amorphous glibenclamide

Glibenclamide crystals were melted in a crucible by heating on a paraffin oil bath maintained at 185 °C and was solidified by quenched cooling with dry ice. The solid powder formed was characterized by methods 1.4.1-1.4.5 and was placed in a desiccator over silica gel until further use. (Hassan et al., 1991)

1.2 Characterization of glibenclamide.

Glibenclamide as received and amorphous glibenclamide produced were characterized using solid state characterization and analytical techniques according to the following;

1.2.1 X-ray powder diffractometry (XRPD)

The X-ray powder diffraction patterns of glibenclamide were recorded on a Bruker AXS Model D8 Discover. The recorder was operated under the following conditions: CuKα radiation; voltage 40kV; current 40 mA; angle 5-45 °2Θ; increment 0.025 degree/step; scan speed 1 sec/step with scintillation detector.

1.2.2 Differential scanning calorimetry (DSC)

The thermal analysis of Glibenclamide was performed by differential scanning calorimetry (DSC). The DSC thermal analysis was measured from 25°C to 250°C at a heating rate of 10 °C/min. Samples (5 -10 mg) were placed in 40 µl aluminum sample pan and crimped with a pinhole. An empty sample pan was used as a reference.

1.2.3 High performance liquid chromatography (HPLC)

HPLC method for the determination of glibenclamide was modified from Galal et al. (2003) and analytical validation was performed according to the following.

HPLC Chromatographic conditions:

Column	: Hypersil® DBS (C18) column (250 x 4.6 mm), particle size 5 µm equipped with guard column packed with BDS (C18), 5 µm set at an ambient temperature.
Detector	: UV detector at 227 nm.
Injection volume	: 20 µL
Mobile Phase	: 1 % acetic acid in water/acetonitrile (50:50)

Flow rate : 1.4 ml/min.

Mobile phase were filtered through a 0.45 μm Millipore® filter and degassed for at least 30 minutes prior to use.

Analytical Validation of HPLC method

The typical parameters to be considered for analytical validation are specificity, linearity (R^2) and precision (%RSD)

Standard preparation

Twenty-eight milligrams of glibenclamide were weighted in to a 250-ml volumetric flask, dissolved in methanol and diluted to volume (concentration of solution = 112 μg/ml). Then, this solution was diluted with methanol to six concentrations (4.5, 9.0, 11.2, 17.9, 22.4 and 33.6μg/ml) which were used as standard solution.

Specificity

The standard preparation was diluted with methanol to obtain the final concentration of 9 μg/ml of glibenclamide. The specificity of the active constituent peaks was determined by the resolution and tailing factor (symmetry factor). The well resolved and symmetric peaks should be obtained.

Linearity

Triplicate injections of solutions containing drug in various concentrations ranging from 4.5 to 33.6 μg/ml of glibenclamide reference standard in methanol were prepared and analyzed. The linear equation of the curve obtained by plotting the peak area at each level prepared versus the concentrations of each standard was calculated using the least square method. The coefficient of

determination (R^2) of the regression line is presented to evaluate linearity; while, the slope and the intercept are essential to contract the equation of the regression line.

Precision

The standard preparation was diluted with methanol to obtain the final concentration of 9 µg/ml. Six replicate injections of this standard solution were analyzed. Percentage of relative standard deviation (%RSD) was calculated for determine the precision.

Sample preparation

Glibenclamide and amorphous glibenclamide powder

Weigh the sample approximately 30 milligrams of glibenclamide in a 100 ml volumetric flask, dissolved in methanol and diluted to volume. Transfer 1.0ml of this solution in to a 25 ml volumetric flask and methanol was added to volume. The concentration of glibenclamide each sample were assayed by HPLC method in triplicate.

Amorphous glibenclamide Tablets

A tablet was placed in mortar and gently ground by a pestel Then, Weigh the sample approximately 30 milligrams in a 100 ml volumetric flask, dissolved in methanol and diluted to volume. Transfer 1.0ml of this solution in to a 25 ml volumetric flask and methanol was added to volume. The concentration of glibenclamide each sample were assayed by HPLC method in triplicate.

1.3 Preparation of amorphous glibenclamide tablets

The equipment used was a tableting machine with an ability to control the compression pressure for producing amorphous glibenclamide tablets. Amorphous

glibenclamide powder 350 mg were placed in ½ inch diameter die and a pressure of 500 psi was introduced for 2 minutes to compact the tablets.

1.4 Solid state stability of amorphous glibenclamide

To know how amorphous glibenclamide interconverted with time and temperature, amorphous glibenclamide was exposed to temperature of 50°C and 60°C for 4 weeks. Over a period of every one week, the samples were collected for physical appearance evaluation and analyzed using XRPD, DSC and equilibrium solubility determination.

1.4.1 X-ray powder diffraction (XRPD)

The X-ray powder diffraction patterns of amorphous glibenclamide powder were recorded on a Bruker AXS Model D8 Discover. The recorder was operated under the following conditions : CuKα radiation; voltage 40kV; current 40 mA; angle 5-40 °2Θ; increment 0.025 degree/step; scan speed 1 sec/step with scintillation detector.

1.4.2 Differential scanning calorimetric (DSC) measurement

The thermal analyses of Glibenclamide were performed by differential scanning calorimetry (DSC).The DSC thermal analysis was measured from 25°C to 250°C at a heating rate of 10 °C/min. Samples (5 -10 mg) were placed in 40 µl aluminum sample pan and crimped with a pinhole. An empty sample pan was used as reference

1.4.3 Surface morphology of glibenclamide tablets

The surface morphology of amorphous glibenclamide tablets were visualized using a digital camera. Samples were placed in black surface.

1.4.4 Equilibrium Solubility of glibenclamide

Equilibrium solubility is measured by shaking an excess of the solid glibenclamide in the presence of the selected solvent, in a sealed container at 37°C±0.5°C temperature. After equilibration, a sample is withdrawn, the solid is filtered out and the clear liquid assayed. This process is repeated until the concentration measured does not rise on successive measurement.

The solubility profile of amorphous glibenclamide was investigated by shaking samples of 100 mg amorphous glibenclamide powder in 200 ml phosphate buffer pH 7.4 in sealed Erlenmeyer flasks at 37°C±0.5°C. The samples are withdrawn, the solid is filtered and the clear liquid assayed at times 1, 3, 6, 9, 18, 30, 42, 54, 72 and 96 hours in triplicate.

In this study the solubility of glibenclamide powder and tablets after storage at 50°C and 60°C at different time intervals were evaluated. Sample of 5 mg glibenclamide was placed in 10 ml phosphate buffer (pH 7.4) in an Erlenmeyer flask. The flask was agitated at 100 strokes/minute in a thermostatically controlled shaking water bath and adjusted to 37±0.5°C. A preliminary review, Hannsan (1991) was presented that at 48 hours the solubility of glibenclamide was reached to the saturated solubility. Hence for this experiment, the sample was collected after 48 hours and filtered through a 0.45μm Millipore® filter. Samples were prepared in triplicate. The concentration of glibenclamide in each collected sample was assayed in duplicate by HPLC method.

1.4.5 Chemical degradation determination of glibenclamide

The chemical degradation of glibenclamide may have occurred when glibenclamide was melted and rapidly cooled in order to produce an amorphous solid. Percentage of chemical degradation of solidified glibenclamide powder was analyzed by using HPLC and calculated as following;

$$\% \text{ drug degradation} = \frac{(\%\text{recovery at time 0} - \%\text{recovery after storage})}{\%\text{ recovery at time 0}} \times 100 \text{----- Eq. 2}$$

The same equation was also used to determine the extent of chemical degradation of amorphous glibenclamide tablets after storage at different temperatures. In this case, using raw material glibenclamide as a reference for calculated the degradation then, %recovery at time 0 in the equation is 100%.

2. Acetylsalicylic acid and salicylic acid

This experiment was aimed to study the chemical degradation of aspirin due to temperature and moisture. Commercial aspirin tablets (British Dispensary, L/C No. I10429) were used as the starting material for the experiments.

2.1 Analysis of aspirin and salicylic acid content in tablets by high performance liquid chromatography (HPLC)

HPLC method was used for the determination of aspirin and salicylic acid contents in the commercial tablets and was modified from Jerry Fogel, et al. (1984) and analytical validation was performed according to the following;

Column : Hypersil®DBS (C18) column (250 x 4.6 mm), Particle size 5 μm equipped with guard column packed with BDS (C18), 5 μm set at an ambient temperature.

Detector : UV detector at 295 nm

Injection volume : 20 μL

Mobile Phase : Water-acetonitrile-phosphoric acid (76:24:0.5)

Flow rate : 1.4 ml/min

Mobile phase were filtered through a 0.45μm Millipore® filter and degassed for at least 30 minutes prior to use.

Analytical Validation of HPLC method

The typical parameters to be considered for analytical validation of HPLC are specificity, linearity (R^2) and precision (%RSD).

Standard preparation

Aspirin standard solution

Five hundred milligrams of working standard aspirin were weighted in to a 50-ml volumetric flask, dissolved in acetronitrile-methanol-phosphoric acid (92:8:0.5) and diluted to volume (concentration of solution = 10 mg/ml). Then, this solution was diluted with acetronitrile-methanol-phosphoric acid (92:8:0.5) to six concentrations (1, 1.6, 2, 3, 4 and 5 mg/ml) which were used as standard solution for aspirin.

Salicylic acid standard solution

Thirty milligrams of working standard salicylic acid were weighted in to a 100-ml volumetric flask, dissolved in acetronitrile-methanol-phosphoric acid (92:8:0.5) and diluted to volume. This solution was diluted with acetronitrile-methanol-phosphoric acid (92:8:0.5) obtain the final concentration 30 μg/ml. Then, this solution was diluted with acetronitrile-methanol-phosphoric acid (92:8:0.5) six concentrations (3, 6, 9, 12, 15 and 18μg/ml) which were used as standard solution for salicylic acid.

Specificity

The standard preparation was diluted with acetonitrile-methanol-phosphoric acid (92:8:0.5) obtain the final concentration 3 mg/ml of aspirin and 6 µg/ml of salicylic acid. The specificity of the active constituent peaks was determined by the resolution and tailing factor (symmetry factor). The well resolving from the other peaks and symmetry peaks should be obtained.

Linearity

Triplicate injections of solutions containing drug in various concentration ranging from 1 to 5 mg/ml and 3 to 18 µg/ml of aspirin and salicylic acid reference standard as respectively, in acetonitrile-methanol-phosphoric acid (92:8:0.5) were analyzed. The linear equation of the curve obtained by plotting the peak area at each concentration level versus the concentrations of each standard was calculated using the least square method.

Precision

The standard preparation diluted with acetonitrile-methanol-phosphoric acid (92:8:0.5) to obtain the final concentration 3 mg/ml of aspirin and 6 µg/ml of salicylic acid. Six replicate injections of this standard solution were analyzed. Percentages of relative standard deviation (%RSD) were calculated to determine of precision.

Tablets Sample preparation

Three tablets were placed in mortar and gently ground by a pestel Then, Weigh the sample approximately 120 mg in a 10 ml volumetric flask and the dilution solvent (acetonitrile-methanol-phosphoric acid (92:8:0.5)) was added to volume. The sample was shaken for 15 min and filtered through a 0.45 µm Millipore® filter. Three milliliters of sample transfer to 10 ml volumetric flask and

the dilution solvent (acetonitrile-methanol-phosphoric acid (92:8:0.5)) was added to volume. The concentration of aspirin and salicylic acid in each sample were assayed by HPLC method in triplicate.

2.2 Stability of aspirin tablets

To know how the aspirin degraded with time and temperature, aspirin tablets were kept in a desiccator controlled at 75 % relative humidity and then placed in a hot air oven at a controlled temperature for 8 weeks. The effect of temperature on aspirin degradation was evaluated after exposed to room temperature 28±2°C, 37°C, 45°C, 50 °C and 60°C. The samples were evaluated every 2 weeks using HPLC. In order to calculate how much aspirin had degraded, the aspirin concentration was compared to the amount of salicylic present.

2.2.1 Calculations

In addition, the percentage of free SA in the tablet was calculated using with the following equation:

$$\% \text{ of free salicylic acid} = \frac{[SA] \times V \times (DF) \times 100}{(\text{mg Aspirin})} \quad \text{----------Eq. 3}$$

Where, mg Aspirin = Sample weight, approximately 120 mg in this case.

It is easier to measure the increasing concentration of the degraded product, salicylic acid (SA), than it is to measure the decreasing aspirin(A) concentration. Therefore, this experiment followed the appearance of SA, and relates the SA concentration to the concentration of A. One mole of SA is produced when one mole of A is degraded; so, using the ratio of the molecular weights of A to SA, we can determine the weight of A degraded for each mg of SA produced.

$$\frac{180.16\frac{g\,A}{mol}}{138.12\frac{g\,SA}{mol}} = \frac{1.304\,mg\,A}{1\,mg\,SA} \quad \text{---------- Eq. 4}$$

Therefore, each milligram of SA present represents the degradation of 1.304 milligrams of A. Since the amount of A initially is known and since the amount of A, which has degraded, can be determined, the amount of A remaining can be calculated.

$$A_{dgrd} = [SA] \times V \times (DF) \times 1.304 \quad \text{---------- Eq. 5}$$

Where,

A_{dgrd} = the mass of A degraded, (mg)

[SA] = concentration of SA (mg/ml)

V = Total volume of SA solution (ml), 10 in this case.

DF = Dilution Factor, 10/3 in this case.

The Amount of A remaining for each sample time calculated as following:

$$A_{rem} = A_0 - A_{dgrd} \quad \text{---------- Eq. 6}$$

Where,

A_{rem} = the mass of A remaining (not yet degraded), (mg).

A_0 = the initial amount of A in the experiment, (mg).

A_{dgrd} = the mass of A degraded, (mg)

The kinetics of aspirin degradation can be referred to a first order reaction. The curves were obtained from plotting ln ($[A_{rem}] / [A_0]$) as a function of time.

$$\ln([A_{rem}] / [A_0]) = - k_{app} * t \quad \text{----------Eq. 7}$$

Where, k_{app} is the apparent rate constant.

The first-order rate constant plots (ln ($[A_{rem}]$/ $[A_0]$) versus time) theoretically include the initial data point (0, 0) (i.e. at the start of the experiment no degradation should occur). There options are to be considered in such a case:

1. The built in function in Excel could be used to force the regression through (0, 0)
2. An initial point (0, 0) could be added to the data used to calculate the regression line.
3. Do a regression without regard to the point (0, 0).

However, in our experiment, the time measurement cannot correspond to the exact start of the reaction, especially because additional time is required for the sample to reach the desired reaction temperature. Therefore, (0, 0) is not a real initial data point and was not used in the regression analysis for k_{app}.

3. Encapsulation of Poly-N-isopropylacrylamide and red cabbage color with calcium alginate.

One gram of sodium alginate was dissolved in 100 ml of distilled water to form the solution of the encapsulation material. The final alginate concentration was 1.0% (w/v). PNIAAM 0.1 gram was added to the polymer solution. Red cabbage extract (200 mg), a model edible pH indicator, was dissolved in 2mL of distilled water and added to the polymer solution. The solution was sprayed into 500 ml of $CaCl_2$ aqueous solution (0.1 M) and the resultant solid beads were stirred for 5 minutes. To remove unentrapped color, the beads were washed several times with distilled water. (Kim et al., 2005)

However, the red cabbage extract showed low entrapment efficiency in the beads due to its water soluble property. The red cabbage extract had to be prepared in other forms which were to dissolve in water insoluble carrier before entrapping in the alginate-PNIAAm beads.

There were two methods which were used to produce the red cabbage extract in term of water insoluble form as follows;

3.1 Encapsulation of red cabbage extract with ethylcellulose polymer by precipitation method.

Ethylcellulose (2g) was dissolved completely in 100 ml acetone in glass beaker and red cabbage extract powder (500 mg) was dispersed into ethylcellulose solution. The mixture was stirred at fixed stirring rate (300 rpm) over 10 min, then poured into 100 ml liquid paraffin which had been previously cooled to 25°C. The emulsion was heated to 45°C at a constant temperature and was continually stirred until the acetone was removed completely by evaporation. Then, 50 ml of hexane was added to the suspension of microspheres and later separated by filtration, washed twice with 50 ml hexane, and dried at 40°C for 12 hours. The microspheres were dispersed in to sodium alginate solution with PNIAAm. Calcium alginate gel beads were prepared by spraying the suspension into calcium chloride solution.

3.1.1 The evaluation of temperature sensitive indicator microspheres prepared by precipitation method.

The microspheres were added in 0.1 normal potassium bicarbonate (pH 9) and kept at two temperatures which were at 25 $\pm$ 2 °C and 80°C for 24 hours. The color change of the microspheres was observed.

3.2 Encapsulation of red cabbage extract by emulsification method

Red cabbage color water in oil emulsion prepared using water containing red cabbage extract 0.1%w/v and low-HLB surfactant (span 80) at a concentration range of 0.1% - 40% w/v solution in isopropyl myristate at an equal volume ratio. The emulsion was subjected to strong stirring of 1000 rpm for 5 minutes to disperse homogeneously. The preparation method consisted of the following steps: disperse of the emulsion into an aqueous sodium alginate solution with PNIAAm to

give water in oil in water emulsion, preparation of calcium alginate gel beads by spraying a emulsion into calcium chloride solution to solidify.

3.1.2 The evaluation of temperature sensitive indicator microspheres prepared by emulsification method.

The microspheres were added in 0.1 normal potassium bicarbonate (pH 9) and kept at two temperatures which were at 25 $\pm$ 2 °C and 80°C for 24 hours. The color change of the microspheres was observed.

4. Preparation of thin films as drug degradation indicator

4.1 Preparation of hydroxypropyl methylcellulose (HPMC) thin films

The films were prepared by layering method and each films were dried at ambient temperature separately and finally assembled together using HPMC solution as adhesive. Each films layer prepared as follows;

The films were prepared by weighing 20 grams of polymer solution in a glass plate diameter 9.5 cm and the dried film samples were cut into squares (2cmx2 cm) then the thickness and the percent moisture absorption were determined.

4.1.1 Preparation of HPMC thin films containing poly-N-isopropylacrylamide (PNIAAm)

Poly-N-isopropylacrylamide powder were sieved through mesh size number 60 (0.25 mm) for selecting particle size of not more than 250 μm. Dissolving poly-*N*-isopropylacrylamide from 0.00-1.00%W/W in the film forming solution (5% w/w HPMC with 2%w/w PEG 400 in water). The film solutions were cast at room temperature for 3 days.

4.1.2 Preparation of HPMC films containing red cabbage color.

Red cabbage extract concentration in the film forming solutions (5% w/w HPMC with 2%w/w PEG 400 in water) varied from 0 – 0.5 % w/w of film solution. Adjust pH of film solution to pH 3 using hydrochloric acid 1 Normal (HCl 8.5 ml in water 100ml). The pH of the film solution was determined by comparing the color of the litmus paper to the scale of colors which was printed on its packet. The film solutions were cast at room temperature for 3 days and were protected from light prior to testing.

4.1.3 Preparation of HPMC Alkaline films.

The 5% w/w HPMC in water containing 2% w/w PEG400 was prepared. The film solution contained 0.1 – 1 % w/w of the potassium bicarbonate. The pH value of polymer solution was approximately pH 9. The film solutions were cast at room temperature for 3 days.

4.2 Characterization of thin films as drug degradation indicator

Thin films were characterized according to the following;

4.2.1 Thermal Analysis of thin films

The thermal analyses of various film compositions in 4.1.1 were performed by differential scanning calorimetry (DSC). Samples (5 -10 mg) were placed in 40 µl aluminum sample pan and crimped with a pinhole. An empty sample pan was used as reference. The DSC thermal analysis was measured from 20°C to 70°C at a heating rate of 5 °C/min.

4.2.2 Thin film thickness measurement

The thickness of each dried film was monitored. Thin film thickness measured using a micrometer or vernier caliper. At least six measurements of the thickness were made at different positions. Thickness will be evaluated on the average of all measurements.

4.2.3 Moisture sorption

The moisture sorption of the HPMC with various composition film were determined. The dried film samples were cut into squares (2cm x 2cm) then the sample was weighed to obtain the initial weight. The samples were conditioned in a container at 100% RH for 48 hours. After 48 hours, the samples were removed from the container, dried by wiping with filter paper, and then weighed immediately to obtain the final weight. The percentage of sample weight increased was calculated as follows;

$$\text{Moisture sorption (\%)} = \frac{\text{final weight} - \text{initial weight}}{\text{initial weight}} \times 100 \quad \text{----------Eq. 8}$$

4.2.4 Diffusion of red cabbage extract through poly-n-isopropylacrylamide barrier films to react with alkaline layer

To investigated the temperature-sensitive indicator from PNIAAm, the diffusions was observed at 60°C, which affect the polymorphisms and degradation for glibenclamide and aspirin as the studied before respectively. The reaction was evaluated by detecting the change of films color and comparing with reference standard films.

Scanning the thin films By Lexmark X83 scanner, then open a picture at a time by using image editing software, Photoshop® and separate thin films sample

to ten sections with the eyedropper Tool. For each square sections, the Lightness, a and b were obtained from info Palette.

The a*values were compared between sections. The Method assumed that the color distribution was symmetrical throughout the sections and showed different between samples.

Methods used to compare color change data are, Statistical Methods (Repeated Measures Design Multivariate Approach (MANOVA)) and Model Independent Methods (Difference factor (f_1), Similarity factor (f_2))