CHAPTER 2

MATERIALS AND METHODS

2.1 Biological materials

Fresh latex clone : RRIM 600, GT 1 and PB 5/51 were bought from rubber plantation in Rayong province, Thailand.

β-carotene type III from carrot, (+)-α-tocopherol mixed isomer type VI from vegetable oil and polyphenol oxidase from mushroom were bought from Sigma.

2.2 Chemicals

Most chemicals used in the present investigation were of analytical or reagent grade. Phenol reagent was prepared in the Department of Biochemistry's laboratory by the method of Ciocalteu (1927).

Acetic acid, phosphoric acid, silica gel for thin layer chromatography, silica gel 60 for column chromatography, sodium acetate, sodium chloride, and tyrosine were bought from E. Merck.

Ammonium hydroxide was bought from Riedel-de Haën.

Catechol and copper sulfate were bought from Sigma Chemical Company.

Formic acid, hydrochloric acid, lithium sulfate, sodium carbonate and sodium molybdate were bought from BDH Chemical Ltd.

Potassium sodium tartrate was bought from May & Baker Ltd.

Sodium tungstate and Tris (hydroxymethyl)-aminomethane were bought from Fluka Chemical Company.

Triton X-100 was bought from Packard company.

Compounds used in vulcanization of raw rubber were of industrial grade and kindly provided by Banpan Research Laboratory Company.

Vulcanizing ingredients : 2-2 Di - benzothiazyl disulfide, 22 cp 46 diethylene glycol, Hisil 233S, 2 - mercaptobenzothiazole, Shellflex, stearic acid, sulfur, tetramethylthiuram, wax and zinc oxide.

2.3 Solvents

Acetone was bought from BDH chemical Ltd.

Chloroform, diethylether and ethanol were bought from E. Merck.

Hexane was bought from Farmitalia Carls Erba.

Methanol was bought from Mallinckrodt.

2.4 Instruments

Autoclave model HA-30, Hirayama Manufacturing Co., Japan.

Durometer, Material testing 3100 (shore A) model 7206, Zwick, Germany.

Hydraulic press model TEE 120, Dalityan hydaulic machine industrial Co., Ltd., Taiwan

Micrometer model SM-114, Teclock Co., Japan.

Mooney viscometer model SMV - 201, Shimadzu, Japan.

Oven model UL-80, Memmert, Germany.

pH meter model PHM 83 autocal, Radiometer, Denmark.

Rheometer model 100S, Monsanto, U.K.

Rotatory Evaporator model RE 111, Büchi, Switzerland.

Spectrophotometer model spectronic 2000, Bosch & Lomb, U.S.A.

Spectrophotometer model spectronic 20D, Bosch & Lomb, U.S.A.

Standard Lovibond comparator disc model 4/19 A and 4/19 B, Tintometer Ltd., England.

Two-roll mill model LRM 200, Lab tech engineering Co., Ltd.

Stress and strain testing machine model 1011, Instron, U.S.A.

Ultracentrifuge model L8-70, Bechman Instrument Inc., U.S.A.

2.5 Preparation of fresh latex

Three clones of rubber were used in this study : RRIM 600, GT 1 and PB 5/51. Fresh latex of each clone was divided into two parts, the first part was added with ammonium hydroxide to the final concentration at 0.25% for lipids and polyphenols determination, the second part for polyphenol oxidase analysis (modified from Karunaratne, 1970) was added with 0.08 M Tris-HCl buffer in 1.0 M Sucrose, pH 7.0, in 3:1 ratio (buffer : latex) and stored at 0°C.

2.6 Determination of dry rubber content (DRC) (ประไพ สุทิน และคณะ, 2531)

Dry rubber content is the weight of rubber in 100 g of latex. It is determined by weighing about 5 g of latex which was then coagulated with 2% formic acid and left until liquid phase was clear. The coagulum was sheeted out by a two-roll mill and washed with excess water. The rubber sheet was then dried in an oven to a constant weight.

$$\% \text{ DRC} = \frac{\text{weight of dry rubber} \times 100}{\text{weight of latex}}$$

2.7 Preparation of air dried sheet rubber (modified from วราภรณ์ ขจรไชยกุล, 2532)

The acid coagulated rubber was prepared by adding 2% formic acid into the latex at equal volume. The mixture was left overnight for complete coagulation. The resulting coagulum was pressed into a thin sheet and then washed with water. The rubber was dried in an oven at 60°C.

2.8 Extraction of lipids and polyphenols (Hasma, 1984)

Fresh latex was separated into two fractions by adding five volumes of chloroform-methanol (2:1, v/v) with continuous shaking. This mixture was left at 4°C overnight to precipitate out as much rubber as possible in the bottom fraction. The coagulum was later removed to prepare an air dried sheet rubber. The solvent fraction was transferred into a separatory funnel, washed with 1/5 of its volume with 0.6% sodium chloride solution in order to separate the aqueous phase with water soluble materials from the chloroform fraction containing total lipids (Folch et al., 1957). The mixture was left standing until clear separation was observed. The upper aqueous fraction was collected to determine the polyphenols content, and the lower chloroform fraction was concentrated in a rotatory evaporatory to dryness, weighed for the total extracted lipids and redissolved in hexane-diethylether (95:5, v/v) for further separation.

2.9 Separation of carotenoids and tocotrienols fraction

2.9.1 Separation by column chromatography (Hasma, 1984)

Carotenoid pigments and tocotrienols were firstly separated on a silica gel (15 g, preactivated at 120°C, 2 h) column preequilibrated with hexane - diethylether (95:5). Total extracted lipids (0.10-0.20 g) was loaded to in the same solvent. The column was subsequently eluted with 100 ml each of hexane - diethylether (95:5), hexane - diethylether (75:25) and diethylether. The eluents were collected separately and then concentrated.

Extraction of total lipids from fresh latex is always accompanied by rubber hydrocarbon, which has to be removed prior to the determination of total lipids. The 95:5 chromatographic eluent containing rubber was concentrated and extracted with acetone. The acetone-insoluble rubber will be precipitated out leaving the esters and carotenoid pigments in the solution. The weight of total extracts minus the weight of rubber gave the weight of total lipids.

2.9.2 Separation by thin-layer chromatography (TLC) (Work and Work, 1975)

A uniform slurry of silica gel G powder in twice its weight of distilled water was prepared by shaking the mixture well in a glass-stopped round-bottomed flask. The mixture was then spread on 20x20 cm cleaned glass plates to a thickness of 0.25 mm with a Desaga spreader. The plates were first air-dried at room temperature before being activated in an oven at 120°C for about 2 h.

Each of column chromatographic eluent was spotted on the TLC plates. The plates were placed in a chromatographic tank, lined with

filter paper and containing about 100 ml of hexane : diethylether : acetic acid (90:10:1). Carotenoid pigments can be detected visually as orange or yellow spots. Tocotrienols were detected as blue fluorescent spot when viewed under ultraviolet light.

2.10 Determination of total carotenoids (Zscheile et al., 1942)

The amount of carotenoids was estimated by dissolving total carotenoids fraction in the known volume of hexane and the absorbance was measured at the wavelength 450 nm. The concentration of total carotenoids were then determined with reference to β-carotene standard as follows :

$$C = \frac{A}{\Sigma l}$$

where C = Concentration of total carotenoids in grams per 100 ml

A = Absorbance at 450 nm

Σ = Absorption coefficience of β-carotene at 450 nm in g/100 ml is 2575

l = Thickness of solution layer in centimeter

2.11 Determination of total tocotrienols (Whittle et al., 1966)

The amount of total tocotrienols was estimated by dissolving total tocotrienols in the known volume of ethanol and the absorbance was measured at the wavelength 295 nm. The concentration of total tocotrienols was calculated with reference to γ-tocotrienol as in 2.10, where the absorption coefficience of γ-tocotrienol at 295 nm is

104.1 g/100 ml.

2.12 Determination of total polyphenols (Hanover et al., 1979)

The amount of total polyphenols was determined spectrophotometrically with Folin-Ciocalteu reaction. By mixing 1.0 ml of the top aqueous fraction containing polyphenols from 2.8 with 5.0 ml of alkaline copper reagent and then stand for 10 min. The mixture was added with 0.5 ml diluted phenol reagent (1:2) to stop the reaction and color complex, and left standing for 30 min. The absorbance of reaction mixture was measured at the wavelength 650 nm. The concentration of total polyphenols was calculated and expressed as tyrosine as follows:

$$\text{Concentration of polyphenols} = \frac{\text{Absorbance at 650 nm}}{\text{Slope of tyrosine standard graph}}$$

2.13 Determination of polyphenol oxidase, PPO (modified from Karunaratne, 1970)

Throughout the extraction process the temperature was kept around 0-10°C. Fresh latex collected and diluted with Tris buffer (2.5) was separated into 3 fractions by centrifugation at 120,000 g, 4°C for 20 min in an ultracentrifuge (Beckman model L8-70) in a swing out roter SW 50.1. Each fraction was added with 0.01% Triton X-100 and stirred well to disrupt the membrane bound organelles and to extract the enzyme PPO into buffer. The suspensions were centrifuged and the supernatant fraction was assayed for PPO activity by measuring initial rate of the increase in absorbance at 410 nm with a double beam spectrophotometer (Spectronic 2000) at 30°C. The sample cuvette contained 1.0 ml of

0.2 M sodium acetate buffer pH 5.6, 1.0 ml 10 mM catechol, 0.8 ml distilled water, and 0.2 ml enzyme solution. The reference cuvette contained 1.0 ml of the same buffer, 1.0 ml 10 mM catechol and 1.0 ml distilled water. Under these conditions, initial rate of enzyme catalyzed reaction was a linear function of time for 2 min. One unit of PPO activity was defined as the potency of enzyme that increased in absorbance by 0.001/min.

2.14 Supplementation of carotenoid, tocotrienol, polyphenol and polyphenol oxidase into rubber latex

Natural rubber latex contains a certain amounts of each factor, which the average values had been estimated by 2.10-2.13, from these baseline conditions the amount of each substance added into the latex separately was one fold + 2 S.D. of its existence in natural condition. The natural latex was divided into 5 parts. The first part was the control of each set, the other 4 parts were added with one of the 4 verifying indicators; carotenoid, tocotrienol, polyphenol and polyphenol oxidase. The treated latex was stirred with a glass rod for 6 h at room temperature (28°C) and then coagulated with 2% formic acid to prepare an air dried sheet as described in 2.7.

2.15 Determination of physical properties of raw rubber

2.15.1 Determination of color index (ASTM D3157, 1988)

Take a test portion of about 30 g from the homogenized raw rubber and pass not more than 3 times (doubling the sheet between passes) between a two-roll mill at room temperature with the nip setting for final sheet thickness of 1.6 to 1.8 mm. Immediately doubled sheet

which is 3.2 to 3.6 mm thick was punched as two pellets and pressed in the mold between 2 sheets of cellophane using mold plates at not less than 3.5 $mN/_{n}^{2}$ pressure on the mold surface for 5 min at 150°C. The color index of rubber specimen was matched with that of the standard glasses to the closet color index (Lovibond index).

2.15.2 Determination of the Mooney viscosity (ASTM D1646, 1988)

The rubber specimen (150±5 g) was passed between a two-roll mill having a roll temperature of 70±5°C and having a distance between the rolls of 2.5±0.1 mm continuously 10 times. About 27±3 g of homogenized specimen was cut into two equal portions, each of approximate thickness 6 mm that fit the die cavity below and above the roter held in the center by a central hole in one of test piece. The specimen was preheated for 1 min before starting the motor. The rotor was then started and recorded the initial dial gauge reading immediately. The dial gauge reading were recorded at 1 min interval. The viscosity of the rubber was taken as the dial reading at the end of 4^{th} min from the instant when the motor was started.

The viscosity was reported as the viscosity number, the roter size (L for large), the number of minutes for warming up in the machine (1 min), the number of minutes at actual test (4 min) and temperature (100°C). A Mooney viscosity of 50 is reported as 50 ML (1+4) 100°C. One Mooney unit is equivalent to a torque of 0.083 Nm.

2.16 Cure characteristic of the compound rubber (Marsden, 1978)

To determine whether addition of any non-rubber ingredient affect the processibility of rubber, a compounding formulation (Table 2.1) for outsole of shoe was selected to compare the cure characteristic among clonal rubbers plus minus additives.

Table 2.1 The compounding formulation chosen for assessing the cure behavior of natural rubber

Natural rubber	100.0 g
Hisil 233S	45.0
Zinc oxide active	3.0
22 cp 46	0.3
Wax	1.0
Stearic acid	1.2
DEG	3.5
Shellflex	1.5
Sulfur	2.0
MBTS	0.78
MBT	0.2
TMTM	0.12

Hisil 233S (SiO_2) is precipitated silica, an reinforcing filler

22 cp 46 is 2-2 methylene bis (4-methyl-6-P-butyl phenol), an antioxidant

DEG is diethylene glycol, a depressor of surface active of SiO_2

MBTS is 2-2 di-benzothiazyl disulfide, an accelerator

MBT is 2-mercaptobenzothiazole, an accelerator

TMTM is tetramethyl thiuram monosulfide, an accelerator

2.16.1 Preparation of test sample

The rubber specimen (100 g) was first blended on a two-roll mill and then mixed with the vulcanizing ingredients. The order of addition was the mixed suspension of Hisil 233S, DEG, Shellflex and stearic acid followed by the mixed suspension of wax, 22 cp 46 and zinc oxide, left the sample until cooled, and then added MBTS, MBT, TMTM and finally sulfur. The final blend was sheet out and folded onto itself. The folded sheet should give a thickness of approximately 5 mm.

2.16.2 Measurement of cure characteristics

The cure characteristics of the mixed samples were run on a Monsanto rheometer (model 100S) for 10 min at 155°C with the disc oscillating at 3° arc. The test sample was loaded on the top of the oscillating disc and the die immediately closed. The samples were then cured in a heated press in their respective mold for the time to reach optimum cure based on the rheometer graph obtained. From the rheometer curve recorded, all the necessary readings were determined as illustrated earlier.

2.17 Testing of rubber vulcanizates

The effect of increasing carotenoids, tocotrienols, polyphenols or polyphenol oxidase contents on the properties of the rubber vulcanizates were investigated by comparing the following properties; the tensile strength, % elongation at break, 300% modulus, tear strength, hardness and specific gravity. In all the tests of the vulcanizates, the compound rubber was firstly prepared according to Table 2.1 and then cured to its optimum state and then prepared as testpiece for measuring physical properties.

2.17.1 Tensile strength, % elongation and 300% modulus test (ASTM D412, 1989)

Five dumbbell testpieces (Figure 2.1) were cut out from the vulcanized rubber by punching with die using a single stroke of a press. A reference of length 2.0 cm was marked and the thickness of the test piece measured along the reference length by a micrometer dial gauge.

The two ends of the testpiece were clamped into the two grips of the testing machine. The test piece was stretched at a constant rate of traverse of the moving grip of 500±50 mm. The force required to stretch the sample to 300% of reference mark length and to breakage were recorded and calculated as follows :

$$\text{300\% modulus} = \frac{\text{Force at 6.0 cm (kg)}}{\text{Cross-sectional area (cm}^2\text{)}}$$

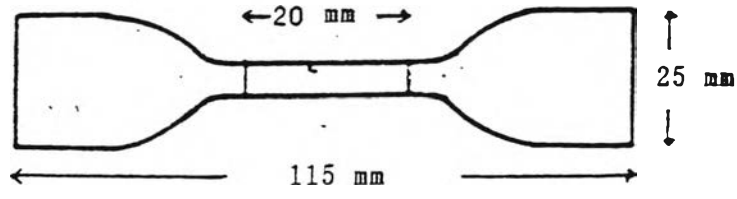
$$\text{tensile strength} = \frac{\text{Force at break (kg)}}{\text{Cross-sectional area (cm}^2\text{)}}$$

$$\text{\% elongation at break} = (\text{length of reference mark at break}-2)\times 50$$

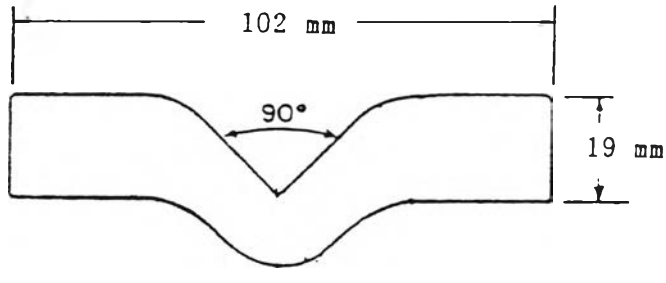
2.17.2 Tear strength test (ASTM D624, 1989)

Five test pieces for tear resistant were cut out from vulcanized rubber by punching with die using a single stroke of press. The thickness of the test piece was measured by micrometer dial gauge.. The highest force required to tear the test piece was recorded and calculated as follows :

$$\text{Tear strength} = \frac{\text{Highest force (kg)}}{\text{Thickness of test piece (cm)}}$$



a)



b)

Figure 2.1 Shape of test piece

a) for tensile strength, % elongation at break and 300% modulus test

b) for tear strength test

2.17.3 Hardness test (ASTM D1415, 1988)

The international hardness test is based on measurement of the penetration of a rigid ball into the rubber specimen under specified condition. Rubber vulcanized was prepared as a flat and smooth sheet having thickness sufficient to fit the gap of type A durometer. The plunger of durometer was pressed with the minor force on to the specimen, the scale was pointed and read as the hardness in shore A at room temperature. The median value of 5 different points distributed over the specimen was recorded.

2.17.4 Specific gravity test

The test method is based on water replacement with test piece. The weight of vulcanized rubber was weighed in the air and then weighed again in the water. The specific gravity of vulcanized was determined as follows :

$$\text{Specific gravity} = \frac{\text{Weight in air (g)}}{\text{Weight in water (g)}}$$