CHAPTER III

EXPERIMENT

The limonin content in preserved lime juice using additives and effects of incorporated air in preserved lime juice were investigated. Additives used in these experiments were potassium metabisulfite and potassium sorbate. Firstly the effects of potassium metabisulfite, potassium sorbate on limonin in full bottle of lime juice were studied by adding 200, 300 ppm of each additive into lime juice, followed by the studies on quality changes during storage, the qualities of the juice were investigated periodically in terms of ascorbic acid content, pH, ^{o}Brix, % acidity, color. Then, the effect of incorporated air in half bottle of preserved lime juice at room temperature and refrigerator temperature was investigated.

3.1 Chemicals

All chemicals used were of reagent grade. Chemicals are tabulated below with manufacturers.

Chemical	Manufacturer
Acetone	AJAX Chemical
L-ascorbic acid	HOPKIN & WILLIAMS
Barium acetate	AJAX Chemical
Butylated hydroxytoluene	Koch-light lab

Chemical	Manufacturer
Chloroform	HOPKIN & WILLIAMS
Ethanol	Riedel DEHAEN
Ethyl acetate	May & Baker
2,4-Dinitrophenylhydrazine	HOPKIN & WILLIAMS
2,6-Dichlorophenolindophenol	MERCK
Metaphosphoric acid	Riedel DEHAEN
Perchloric acid	HOPKIN & WILLIAMS
Potassium metabisulfite	May & Baker
Potassium sorbate	MERCK
Silica gel "G"	MERCK
Stannous chloride	MERCK
Toluene	BDH

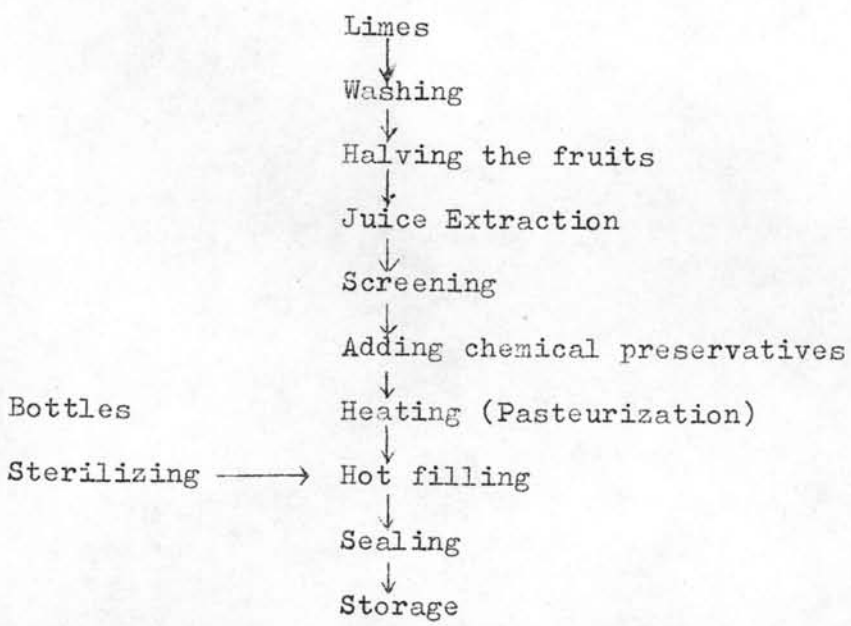
3.2 Instruments

Abbe Refractometer	
Munsel Disk Colorimeter	
pH Meter 7010	Electronic Instruments Limited
UV Spectrophotometer	Prolabo Paris No. 5348
Thin Film Evaporator	Gallenkamp England

3.3 Preparation of Lime Juice

To obtain treated single strength lime juices, the limes were processed as follows;

The limes were immersed under tap water for 10 minutes then washed and rinsed thoroughly to remove all dirt and contamination. The defect limes were also picked out for rejection. The perfect limes were halved by a sharp stainless knife and the juice was extracted by hand-pressed extractor. The juice was passed through a cheese-cloth to remove rag and seeds; this step should be done as quickly as possible in order to minimise the dissolving of the limonoid bitter principles from the rag and seeds. Chemical preservatives were added and the juice was heated up to 85°C for pasteurization and in activated the enzyme. The heated lime juice was hot filled into 250 cm^3 sterilized bottles. The bottles were stoppered tightly and some were stored at room temperature, some were stored at refrigerator temperature. The quality of the juice was investigated periodically in terms of limonin, ascorbic acid content, pH, °Brix, % acidity, color. From the procedure described above it can be presented in the following processing schemes.



3.4 Method of Analysis

3.4.1 Preparation of Plates for Thin-Layer Chromatography

30 g of Silica gel G were shaken for 1 min. in a stoppered flask with 60 cm^3 of distilled water to give a free-flowing suspension suitable for the preparation of plates with the Desaga Thin Layer Chromatography Apparatus. The silica gel coated plates with 0.25 mm layer thickness were air dried for 20 min., oven dried at 110° for 30 min., and stored in a desiccator.

3.4.2 Preparation of Limonin

Limonin was extracted from citrus seeds. The lime seeds were dried and ground. The ground dry lime seed was defatted with petroleum ether. Limonin was extracted from defatted lime seed with $CHCl_3$ by using Soxhlet-extraction technique, solvent was removed and the residue was recrystallized from methylenechloride with propanol, m.p. 296°. It was chromatographically pure and confirmed by IR spectra (Appendix I)

3.4.3 Preparation of Limonin Dinitrophenylhydrazone

Limonin (100 mg) in warm acetic acid (5 cm^3) was treated with dinitrophenylhydrazine (20 cm^3: 1.2 %) in aqueous perchloric acid (15 %). After 2 hours at room temperature, it was filtered, and recrystallized from n-butanol mp. 305°.

3.4.4 Establishment of Limonin Standard Correlation

A solution of limonin dinitrophenylhydrazone 100 $mg/100\ cm^3$ in ethanol (containing 0.1% of butylated hydroxytoluene) was prepared and 1-10, 15, 20, 25, 30, 35 μl of solution were spotted on a thin-

layer plate. A mixture of toluene and ethyl acetate (1:1) was used as the mobile phase. The plate was developed in a glass tank containing the mixture of solvent, until the solvent front height was about 15 cm. (30 min.) The complete chromatogram was dried and the spots of limonin 2,4-dinitrophenylhydrazone were scraped off into centrifuged tubes (10 cm^3). The tube walls were washed down with acetone to a total volume of 4 cm^3 and the tube contents were stirred and centrifuged. The acetone solutions were decanted carefully into a cuvette and the absorbances were recorded at 363 nm against acetone as reference. A linear relationship ($y = bx + a$) was established between the absorbance (y) of the limonin dinitrophenylhydrazone solution and the amount (x μg) of limonin (Appendix II)

3.4.5 Estimation of Limonin in Lime Juice

100 g of well-shaken juice were weighed into 500 cm^3 stoppered erlenmeyer flask containing 10 g of magnesium sulfate and 100 mg of butylated hydroxytoluene (BHT). The solution was extracted with 3x30 cm^3 of chloroform. For each extraction the solution was shaken mechanically for 30 min. then it was transferred to a 125 cm^3 separatory funnel to separate the aqueous layer. The chloroform extracts were combined, transfered to a centrifuge tube and centrifuged at 3700 rpm. Care was taken to exclude as much solid or emulsified material as possible. The combined chloroform extract was transfered to a round bottomed flask (150 cm^3) and evaporated to dryness at 60°C on a rotary vacuum evaporator. The flask was cooled, ethanol (8 cm^3) was added and the flask was heated gently with swirling then the

solution was transfered to a small round bottom flask (50 cm^3) to which sodium hydroxide (0.1N 6 cm^3) was added. After 60 min.reflux, the alcohol was allowed to evaporate by gentle warming. The cooled aqueous solutions were transfered to graduated centrifuged tubes, and the flasks were rinsed out with 2,4-dinitrophenylhydrazine solution (1.2% 2,4-DNP in 15% perchloric acid; 2 × 2.5 cm^3) and finally with sufficient water to give a total volume of 15 cm^3 in each tube. The mixtures were stirred and set aside at room temperature for 16 hours.

To each tube, sulfuric acid (18N; 1 cm^3) and barium acetate solution (3N; 1 cm^3) was then added, with stirring after each addition. The mixture was centrifuged and the supernatant liquor was discarded. The centrifuged material was washed with water (30cm^3) and after a second centrifugal separation the supernatant liquor was discarded. The tube was inverted and its content was allowed to drain. Ethanol (1.5 cm^3) and chloroform (2.5 cm^3) were then added to the precipitate with stirring and the mixture was again centrifuged. The supernatant liquors were kept in a tightly closed screwcap vial at refrigerator temperature until ready for limonin determination by TLC. Each sample of lime juice was extracted three times.

3 spots of each of the supernatant liquors, 20 μl each were spotted on a pre-coated silica gel TLC plate with a micropipette. The plate was developed in toluene : ethyl acetate (1:1) until the solvent front was 16 cm long (30 min.). The plate was air dried, and the spots corresponding to limonin dinitrophenylhydrazone were scraped off into 3 centrifuged tubes. The acetone was added (4 cm^3)

to each tube and centrifuged. The acetone solution was decanted carefully into a cuvette and the absorbance was recorded at 363 nm against acetone as reference.

3.4.6 Analytical Method for Studying the Changes in Juice Quality during Storage

These methods are applied to lime juice which are described in either the AOAC Method of Analysis (1975) or the Chemical Analysis of Food by Pearson.

Determination of Ascorbic acid

The volumetric determination using 2,6-dichlorophenol-indophenol, as described by Cox and Pearson (1962) was used.

Standard Indophenol Solution

0.05 g of 2,6-dichlorophenolindophenol was dissolved in water and diluted to 100 cm^3 and the solution was filtered. To standardise, 0.05 g of pure ascorbic acid was dissolved in 60 cm^3 of 20% metaphosphoric acid and diluted with water to exactly 250 cm^3. Then 10 cm^3 of this solution was pipetted into a small flask and was titrated with the indophenol solution until a faint pink color persisted for fifteen seconds. The concentration was expressed as mg ascorbic acid equivalent to 1 cm^3 of the dye solution.

Sample Analysis

50 cm^3 of the juice was pipetted into a 100 cm^3 volumetric flask, 25 cm^3 of 20 percent metaphosphoric acid was added as stabilising agent and the solution was made up to the mark with water, 10 cm^3 of the solution was pipetted into a small flask, 2.5 cm^3 of acetone was added and the solution was titrated with the standardised

indophenol solution until a faint pink color persisted for fifteen seconds. The vitamin C content in the sample was calculated as mg per 100 cm^3 of juice. The acetone may be omitted if sulfur dioxide is known to be absent. Its function is to form the acetone-bisulfite complex with sulfur dioxide which otherwise interferes with the titration.

Determination of Acidity

The determination of acidity employed the titration with standard sodium hydroxide (0.1 N) using phenolphthalein as indicator.

10 cm^3 of juice was pipetted into a small flask, 20 cm^3 of distilled water was added and the mixture was mixed thoroughly. 10 cm^3 of the solution was pipetted into a small flask, 2 drops of phenolphthalein indicator was added and the solution was titrated with 0.1 N NaOH. The acidity was calculated as percentage citric acid as it was predominant acid in the lime juice.

Determination of Total Soluble Solid (^oBrix)

Total soluble solid of lime juice was determined by means of Abbe Refractometer.

pH Measurement

The pH value of lime juice was determined by using pH meter.

Color Measurement

Color of lime juice was measured by using Munsell Disk Colorimeter. Due to the lack of colored plates used for measuring the color of lime juice, some of the colored plates used for measuring milk and apricots were selected for this experiment. The

color plates which were used were as follows.

Yellow	5Y 8/12
White	N 9.2/
Grey	N 7/
Orange	10 YR 8/6
Green	5 G 8/6

The result was expressed as percentage of each color which would result in the color similar to the lime juice after blending all the colors with Munsell Disk Colorimeter. Beside the color measurement, the browning of the lime juice was observed by visual inspection. The brown color was ranked as follows:

Browning	0	fresh lime juice color
	+	The changing color cannot be distinguished sharply
	++	
	+++	
	++++	light brown, still to be acceptable
	+++++	slightly unacceptable
	++++++	Unacceptable
	+++++++	Brown color
	++++++++	Deep brown color

The increase in the numbers of plus signs indicates the increase in degree of browning of the juice which is expressed as percentage of color.

3.5 Effect of Potassium Metabisulfite

To study the limonin content and the changes quality of preserved lime juice with potassium metabisulfite in full bottle

(250 cm^3 brown bottle, 0.5 cm from the neck of bottle) stored at room temperature (28°C) and refrigerator temperature (7.5°C), samples were prepared by adding various concentrations of potassium metabisulfite as indicated below

Treatment	ppm of potassium metabisulfite
I_1	0
I_3	200
I_4	300

All of the samples were prepared as described before and then were stored at refrigerator temperature and room temperature for 4 months. The limonin content and changes of the lime juice qualities during storage were determined periodically.

3.6 Effect of Potassium Sorbate

To study of limonin content and the changes of the lime juice qualities during storage at room temperature and refrigerator temperature preserved with potassium sorbate in full bottle (250 cm^3 brown bottle, 0.5 cm from the neck of bottle), samples were prepared by adding various concentrations of potassium sorbate as indicated below

Treatment	ppm of potassium sorbate
II_1	0
II_3	200
II_4	300

All of the samples were prepared as described before and they were stored at refrigerator temperature and room temperature for 4 months. The limonin contents and the changes of the lime juice qualities during storage were determined periodically.

3.7 Effect of Incorporated Air

3.7.1 Effect of Potassium Metabisulfite and Potassium Sorbate

To study the effect of various concentration of potassium metabisulfite and potassium sorbate on the preservation of lime juice in half bottle (250 cm^3 brown bottle) stored at room temperature and at refrigerator temperature, samples were prepared by adding various concentration of potassium metabisulfite and potassium sorbate as indicated below

Treatment	ppm of potassium metabisulfite	ppm of potassium sorbate
I_1	0	-
I_3	200	-
I_4	300	-
II_1	-	0
II_3	-	200
II_4	-	300
III_1	200	300
III_3	300	200

All of the samples were prepared as described before and they were stored at refrigerator temperature and room temperature for 4 months. Changes of lime juice qualities during storage were determined periodically.

3.7.2 Effect of Stannous Chloride in Lime Juice with Incorporated Air

To study the effect of 200 ppm of stannous chloride compared to 200 ppm of potassium metabisulfite and controlled lime juice on the preservation of lime juice in half-bottle (250 cm^3 brown bottle) stored at room temperature and refrigerator temperature, samples were prepared by adding additives as indicated above stored for1 month Changes of lime juice qualities during storage were determined periodically.