CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS AND METHODS

2.1.1) Chemicals

Reagents	**Brand**
Acetone	Carlo Erba
Ascorbic acid ($C_6H_8O_6$)	Merck
Bovine serum albumin	Sigma
Cinnamic acid ($C_6H_5CHCHCOOH$)	Fluka
Coomassie brilliant blue G-250	Fluka
2,6-dichlorophenol indophenol sodium salt hydrates ($C_{12}H_6Cl_2NNaO_2$)	Fluka
Ethanol 95%	Merck
Folin-Ciocalteu's reagent	Merck
Glycine (CH_2NH_2 COOH)	Fisher
Hydrogen peroxide	Carlo Erba
L-phenylalanine($C_6H_5CH_2CH(NH_2)COOH$)	Fluka
Methanol	Merck
2-methoxyphenol (Guaiacol)	Fluka
Phosphoric acid	Fluka
Polyvinylpyrolidone (PVP)	Fluka

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Pyrocatechol ($C_6H_6O_2$)	Merck
Sodium borate	Carlo Erba
Sodium chloride	Merck
Sodium carbonate	Merck
Sodium dihydrogen phosphate	Fluka
Sodium hydroxide	Carlo Erba
Sodium phosphate	Fluka
Trifluoroacetic acid (highly toxic)	Acros
Triton X-100	Fluka

2.1.2) Instruments

Instruments	Brand
Hot air oven protocol	Department of chemistry, Faculty of Science, Chiang – Mai University, Thailand supported by Commission on higher education(MUA),Thailand
Microwave oven	Department of Chemistry, Faculty of Science, Chiang Mai University and Department of Physics, Faculty of Science, King Mongkut's University of Technology Thonburi, Thailand
Centrifuge Model D-78532	Hettich, Germany

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Heating mantle	Whatman, USA
Hot plate stirrer	Clifton, UK
Micropipet	Biohit, Finaland
pH meter	Merohm, Switzeland
Spectronic 21	Milton Roy Company, Canada
Water bath	Heto, Denmark
Blender	Moulinex, UK
Shaker	Gemmy Industrial, Taiwan
Desiccators	-
Crucible	Thomas Scientific, USA
Asher Model SCF1200	Sartorius,Germany
Colorimeter Model Colorqust XE	Hunter Lab, USA
Hand Refractometer	Nippon Optical, Japan
Texture analyzer Model TA-XT2i	Stable Micro System, UK
Aw center Model Novasina AG	Pfäffikon, Switzerland
Evaporator Model recirculating- chiller B-740	BUCHI, Switzerland
Hot oven	Cenco Instruments, USA
UV-Vis spectrophotometer	Perkin Elmer instruments, USA
Mitutoyo Model ID-C112BS	Mitutoyo corperation, Japan

2.1.3) Plant materials and microwave pretreatment

Longan (*Euphoria longana* Lam.) was obtained in early August 2006 from Lumphun Province, Thailand. Fruit of uniform size (25±1 mm) and maturity stage were selected and then seeded, peeled and washed.

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Peeled longan pulp (600 g) was placed in 50 cm diameter glass dish for each treatment. Samples were heated in a microwave oven (Figure 2.1) using 2 different microwave power levels: 450 and 900 W for each of 4 time periods: 0, 40, 60 and 90s. After treatment, the microwave-pretreated sample was carried out in hot air dryer (Figure 2.2) immediately for 3 hr at 70°C and then reduced the temperature into 55°C for 4-5 hr. Dried-longan pulp (Figure 2.3) was then analyzed the enzyme activities (PPO, POD and PAL activities) and the qualities as shown in Figure 2.4.

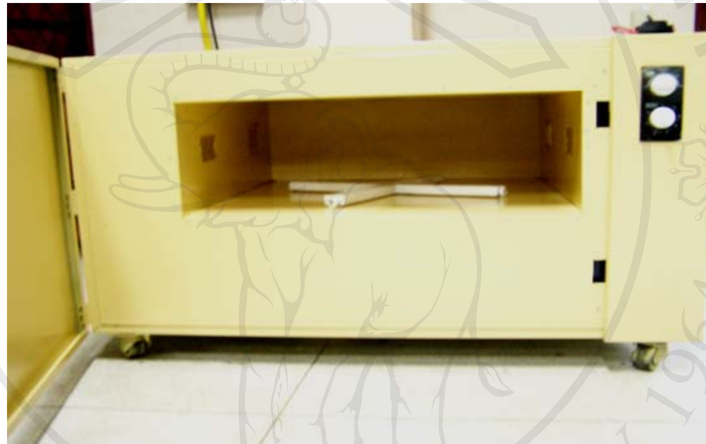

Figure 2.1 The microwave oven used in this study

Figure 2.2 The hot air oven used in this study

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Figure 2.3 The dried-longan pulp pretreated by microwave

Fresh longan

↓

Remove seed and peel

↓

Wash seeded and peeled longan

↓

Pretreat by microwave at 450W and 900W for 40, 60 and 90s

↓

Drying in process by hot air dryer

↓

Dried-longan pulp

↓

- Enzyme activities
  - PPO activity
  - POD activity
  - PAL activity
- Qualities of product
  - Color appearance
  - Firmness
  - Water activity & moisture
  - Titratable acidity
  - Total soluble solid
  - Total phenolic content
  - Fat, Ash, Protein Moisture, Fiber, ascorbic acid &carbohydrate contents
  - Microbiological analysis

Figure 2.4 The procedure of this study

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2.2 EFFECT OF MICROWAVE PRETREATMENT ON POLYPHENOL OXIDASE (PPO), PEROXIDASE (POD), PHENYLALANINE AMMONIA LYASE (PAL) AND PRELIMINARY QUALITY OF DRIED-LONGAN PULP

2.2.1) Effect of microwave pretreatment on polyphenol oxidase, peroxidase and phenylalanine ammonia lyase in dried-longan pulp

2.2.1.1) Determination of PPO activity (Jiang & Li, 2001)

a) Extraction of PPO

To extract polyphenoloxidase (PPO), tissue (3 g) from each of treatment was homogenized in 20 ml of 0.1 M phosphate buffer (pH 6.8) and the homogenate was filtered through 2 layers of cotton to remove cell debris and then centrifuged at 12,000xg (Hettich, Germany) for 20 min at 4°C. The clear supernatant was collected as enzyme extracts.

b) PPO activity assay

PPO activity was assayed according to the method of Jiang & Li (2001) by measuring the oxidation of pyrocatechol. The increasing absorbance at 420 nm incubated at 45°C was recorded every 15 min for 60 min using UV-Vis spectrophotometer (Perkin Elmer instruments, USA). One unit of enzyme activity was defined as the amount that caused a change of 0.001 in absorbance per minute.

c) Protein determination by Bradford method (1976)

The protein content was determined according to the dye-binding method of Bradford (1976) with bovine serum albumin as the standard.

Bovine serum albumin 0.0100 g was dissolved with distilled water and adjusted the total volume to 10 ml in volumetric flask.

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The bovine serum albumin solution was used as a standard solution. The dye reagent contains 0.025g of Coomassie Brilliant blue G-250 was dissolved with 11.75 ml of 95% ethanol and 21.25 ml of phosphoric acid. Total volume of reagent was adjusted to 250 ml. NaCl 0.875 g and dissolved into 100 ml of distilled water in volumetric flask.

The sample extracted 0.05ml of sample extraction from 2.2.1.1(a) added into test tube and then adjusted to 0.60 ml with distilled water, 0.10 ml of 0.1 M NaCl and 4.30 ml of dye reagent were added and mixed. After 20 min, the absorbance of the reactant was read at 595nm. The protein content was then determined by referring to the calibration graph, using bovine serum albumin as standard solution (Table 2.1).

Table 2.1 The calibration graph of bovine serum albumin

Solution	**Test tube no.**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1 mg/ml of BSA standard solution (ml)	-	0.02	0.04	0.06	0.08	0.10	-
Sample extraction (ml)	-	-	-	-	-	-	0.05
Distilled water (ml)	0.60	0.58	0.56	0.54	0.52	0.50	0.55
0.1 M NaCl (ml)	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Dye reagent (ml)	4.30	4.30	4.30	4.30	4.30	4.30	4.30
	Mixed for 20 min						
	Measured at A_{595}						

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2.2.1.2) Determination of POD activity (Lin *et al*., 1988b)

a) Extraction of POD

Dried-longan pulp peroxidase (POD) was extracted by homogenizing the tissue 5 g with 20 ml of 0.1 M phosphate buffer (pH6.8) and the homogenate was filtered through 2 layers of cotton to remove cell debris and then centrifuged at 12,000xg (Hettich, Germany) for 20 min at 4°C. The clear supernatant was collect as enzyme extracts.

b) POD activity assay

POD activity was assayed by the method of Lin *et al*. (1988b), with guaiacol as a substrate in a reaction mixture(3 ml), containing 25 µl enzyme extract, 2.75 ml 0.05 M phosphate buffer (pH 6.8), 0.1 ml of 1% H_2O_2 and 0.15 ml of 4% guaiacol. The increase in absorbance at 470 nm to the guaiacol oxidation was recorded every 2 min for 10 min. One unit of enzyme activity was defined as the amount that caused a change of 0.001 in absorbance per minute.

c) Protein determination

The method followed by 2.2.1.1(c).

2.2.1.3) Determination of PAL activity (Zucker, 1968)

a) Extraction of PAL

The method of Zucker (1968) was used to extract phenylalanine ammonia-lyase (PAL). The tissue (1.0g) was homogenized for 30s in chilled 0.05M phosphate buffer (25 ml; pH6.6) with 0.2 g of Triton X-100. Polyvinylpyrolidone (PVP) (25 mg) was added and the suspension was centrifuged at 12,000xg (Hettich, Germany) for 15 min at 4°C. The supernatant was used as a source of crude enzyme.

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b) PAL activity assay

PAL activity was assayed by a slight modification of the method of Zucker (1968). The reaction mixture consisted of 0.06 M sodium borate buffer (pH 8.8) and crude enzyme. The reaction was started by addition of 11mM L-phenylalanine and then incubated at 37°C for 1 h. The reaction was stopped by addition of 35% trifluoroacetic acid (TFA) and was centrifuged at 5,000xg (Hettich, Germany) for 5 min. The cinnamic acid yield was estimated by measuring the absorbance at 290 nm. PAL activity was expressed in μmol cinnamic acid liberated per gramme of dry weight sample (μmol cinnamic acid $g^{-1}dwt^{-1}$)

c) Protein determination

The method followed by 2.2.1) c).

2.2.2 Effect of microwave pretreatment on preliminary qualities of dried longan pulp

2.2.2.1) Analysis of color appearance

The following quality parameters were evaluated to assess the effect of microwave pretreatment on the dried-longan pulp in term of color, firmness, moisture content and water activity.

Color was assessed with a spectrocolorimeter (Hunter lab, Colorqust XE, USA) by measuring *L*a*b** parameters on the pieces of samples, *L** representing the lightness, *a** for redness to greenness while *b** for yellowness to blueness.

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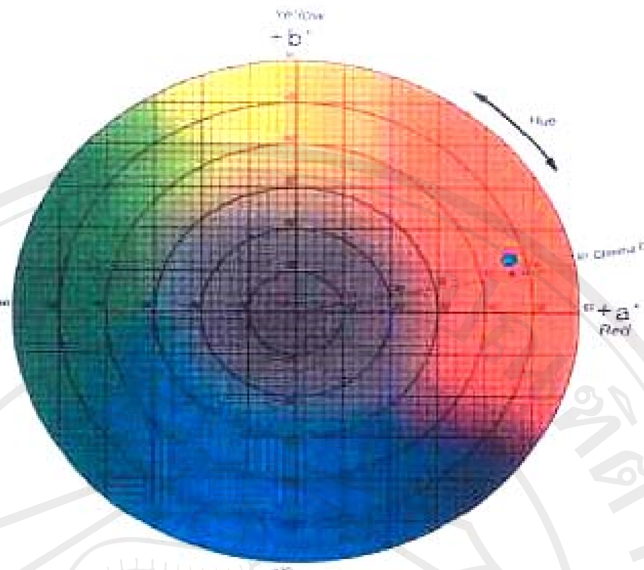

Figure 2.5 Color (CIE) measurements

2.2.2.2) Analysis of firmness

Samples firmness was evaluated with texture analyzer (Stable Micro System, TA-XT2i, UK) using a Kramer shear cell with a blade. The blade speed was set at 40 mm/s and used to cut the sample. The result is determined by measuring the maximum force required to rip the sample. The firmness of all samples was determined in triplicated.

2.2.2.3) Analysis of water activity and moisture content

Water activity (a_w) was analyzed with a_w center (Pfäffikon, Novasina AG, Switzerland) measures the relative equilibrium humidity in % rh and changed with the following formula; a_w = ERH/100. Moisture content of samples was determined using AOAC method (AOAC, 2000) by an oven method at 100-110°C (Cenco Instruments, USA) until constant weight.

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2.2.2.4) Analysis of total soluble solid

The total soluble solid of dried-longan pulp , which homogenized with distilled water, was determined with a hand refractometer (Nippon Optical, Japan). Results were expressed in °Brix (1 °Brix = 1 g of soluble solid / 100 g solution)

2.2.2.5) Analysis of titratable acidity (AOAC, 2000)

5.0 g of dried-longan pulp were homogenized with 10 ml of distilled water and titrated with o.1 N NaOH. The results were calculated as a percentage of citric acid

Titratable acidity (% citric acid) = [(ml of NaOH)*(conc. of NaOH)*100] / [(g of dried-longan pulp)*(ml of sample solution)]

2.2.2.6) Determination of the total phenolic contents (Naczk et al., 1992; Waterman and Mole, 1994)

The amount of total phenolic in samples were determined with Folin-Cio-calteu reagent, tissue(1 g) was soaked in 20 ml of mixed solution containing methanol: acetone: water (7:7:6) and shaked for 2 h (Gemmy Industrial, Taiwan). The solution was centrifuged at 6,000xg (Hettich, Germany) for 10 min and collected for total phenolic determination. Phenolic content of the extract was measured by the method of Lister and Wilson (2001). To 50 µl of each sample extract, 2.5 ml 1/10 dilution of Folin-Ciocalteu's reagent and 2 ml of Na_2CO_3(7.5%w/v) were added and incubated at 45°C for 15 min. The absorbance was measured at 765 nm using UV-Vis spectrophotometer (Perkin Elmer instruments, USA). Results were expressed as milligrammes of pyrocatechol equivalent per gramme of dry weight. Samples were analyzed in triplicate.

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2.2.2.7) Determination of fat content (AOAC, 2000)

The fat content is determined gravimetrically after extraction with hexane. 2 g of dried-longan pulp were extracted with 100 ml of hexane by using sokhlet extraction apparatus. The extraction was continuously boiled until 4 hours. The solution was transferred to evaporating flask (that previously weighed) and evaporated completely on steam bath at 60°C, cooled and weighed. The fat content was calculated as,

$$\% \text{ fat content} = \frac{\text{Weight of flask and fat} - \text{Weight of flask}}{\text{Weight of sample}} \times 100$$

2.2.2.8) Determination of protein content by Kjeldahl method according to AOAC, 2000

The protein content was determined by using Kjeldahl digestion and distillation (Foss, Germany). 2 g of dried-longan pulp were put in a digest tube. Add 2 tablet of Kjeltab catalyst and 20 ml of H_2SO_4. The digest tube was placed in an inclined position in a digestion machine and heated gently at 420°C for 1 h. At end of digestion, digest should be clear and free of undigested materials (clear with light blue-green color). Cool acid digest to room temperature for 20 min and added 75 ml H_2O and then was adjusted as neutral solution with 0.1 N NaOH. The digest tube was transferred to the machine again and continuously distilled. Immediately connect a receiver flask that contains 25 ml of 4% boric acid to distillation, for let liquid drain from condenser tip. After completely distilled, turn off the machine. The receiver flask added 5 drops indicator and titrated with 0.1N HCl. The protein contents are calculated following formula,

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$$\% \text{ protein content} = \frac{1.401 \times 6.25 \times (V1 - V2) \times \text{concentration of titrant}}{\text{g of sample}}$$

V1 = ml of titrant for sample solution; V2 = ml of titrant for blank solution

2.2.2.9) Determination of moisture content (AOAC, 2000)

The determining of moisture content of sample followed by AOAC (2000) method. A crucible was dried in a hot air oven for 30 min, cool in the desicator and weight the crucible. Weigh accurately 2 g of the sample into the crucible. Record the weight of cruclble plus sample. Drying in an air drying oven at 100±30°C for 3 hours, cool in desicator and weight the crucible. The sample in crucible was dried again for several times until the sample has constant, assuming that the loss in weight of the sample on drying is due to the loss of moisture only. The moisture content was calculated as,

$$\% \text{ moisture content} = \frac{\text{loss in the sample weight during drying} \times 100}{\text{Initial weight of the sample}}$$

2.2.2.10) Determination of fiber content (AOAC, 2000)

Weigh 2 g of sample into 500 ml flask; added 200ml of 1.25 % sulfuric acid. The sample solution was boiled on a hot plate for 30 min and then filtered by using a Whatman paper no.54 and washed the residue with boiling water until the sample does not have acid. The residue on the filter paper was transfered to flask contain 200 ml of 1.25% NaOH and boiled for 30 min. The sample was filtered by using Whatman paper no. 41 then washed the residue with boiling water. The sample residue on filter paper transfers to crucible and dried at 600 ± 5°C for 30 min followed by dried in a hot air oven at 130 ± 5°C for 3 hours. Cool in a desicator and weighed,

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then ash the residue dried again at 600 ± 5°C for 2 hours, cool in the desicator and weighed. The crude fiber was calculated as,

$$\% \text{ crude fiber} = \frac{(W_4-W_3+W_2) + (W_5-W_3) \times 100}{W_1}$$

W_1 = Weight of sample

W_2 = Weight of filter paper

W_3 = Weight of crucible

W_4 = Weight of crucible+filter paper+sample after drying

W_5 = Weight of crucible+ash

2.2.2.11) Determination of ash content (AOAC, 2000)

0.5 g of sample was put into a crucible, dried on bunsen lamp until no more black smoke appeared. The crucible plus sample was dried in asher (Sartorius, Germany) at 600°C for 30 min until light gray ash and then cooled in a desicator and weighed. The ash content was calculated as,

$$\%\text{ash content} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

2.2.2.12) Determination of carbohydrate (AOAC, 2000)

Carbohydrate content was determined by calculating the difference of the original sample minus the moisture, protein, ash, fiber and fat contents as,

%carbohydrate content = 100 - (%protein+%fiber+%ash+%moisture+%fat)

2.2.2.13) Determination of ascorbic acid (AOAC, 2000)

1 g of sample was extracted with Meta-phosphoric acid-acetic acid-sulfuric acid solution adjusted pH 1.2 then was diluted with Meta-phosphoric –acetic acid solution. The sample solution was filtered through Whatman no. 1. The filtrate 7

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ml was titrated with 2,6-dichlorophenol indophenol standard solution. At the end point the solution will appear a rose pink color from blue color solution. The vitamin C content was calculated as,

$$\text{Vitamin C content (mg/100g dry weight)} = \frac{(X-B) \times F \times V}{W \times Y}$$

When, X = ml of indophenol standard solution used of sample solution

B = ml of indophenol standard solution used of blank solution

F = concentration of indophenol standard solution

V = total volume of sample extraction

W = weight of sample

Y = ml of sample solution used titration with indophenol standard solution

2.2.2.14) Determination of total plate count and yeasts and molds (FDA, 2001)

Total Plate Count for total viable microorganisms was enumerated using pour plate method on Orange Serum Agar. Incubation was performed at 35 ± 2°C for 48 hours.

The numbers of yeast and molds were enumerated on Potato Dextrose Agar acidified to pH 3.5 with 10% tartaric acid by a pour plating technique. The incubation for total yeast and mold counts was done at 22°C for 5 days.

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2.3 EFFECT OF MICROWAVE PRETREATMENT ON PPO, POD, PAL ACTIVITIES AND THE QUALITY CHANGES OF DREID LONGAN PULP DURING STORAGE TIME

2.3.1) Effect of microwave pretreatment on PPO, POD and PAL activities of dried-longan pulp during storage time

The experiment in 2.2 showed the preliminary studies of effect of microwave pretreatment on the variables qualities of dried-longan pulp in each condition. In this step, amount of dried-longan pulp pretreated with microwave heating was packed into aluminum foil bag and then divided randomly into 2 lots. One lot of sample was stored at 4°C for progressive assessments. Another lot of sample was stored at room temperature. The PPO, POD, PAL activities of these samples was determined every 1 month during storage at 2 different temperatures for 4 months. The data of these results were compared by using the statistical program of SPSS version 13.0 which were performed through an analysis of variance (ANOVA) and Duncan's multiple range test was used for comparing differences between means.

2.3.2) Effect of microwave pretreatment on the quality changes of dried-longan pulp during storage time

The experiment was determined the PPO, POD, PAL activities and total phenolic content changes in dried-longan pulp when pretreated by microwave heating in each condition. In this step, changes qualities of amount of microwave pretreated sample in each condition in the terms of total phenolic content, color appearance, firmness, water activity, moisture content, titratable acidity and total soluble solid would be also determined for a month.

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The data of these results were compared by using the statistical program of SPSS version 13.0 which were performed through an analysis of variance (ANOVA) and Duncan's multiple range test was used for comparing differences between means.

2.4 EFFECT OF DIFFERENT PACKAGING MATERIALS ON PPO, POD, PAL ACTIVITIES AND THE QUALITY CHANGES OF DREID-LONGAN PULP DURING STORAGE TIME

2.4.1) Effect of different packaging materials on PPO, POD and PAL activities of dried-longan pulp during storage time

Although, the experiment showed that microwave pretreatment before drying process could inactivate the PPO, POD and PAL activities in dried-longan pulp**.** However, the storage conditions and type of packaging materials are important factor that influences the discoloration of dried-longan pulp. Thus, this experiment was also considered the effect of different packaging materials on the qualities changes of dried-longan pulp during shelf life. This step, the microwave pretreated and non-treated of dried-longan pulp were packed in 3 different packaging materials, LLDPE/nylon 15 (0.113 mm thickness), OPP (0.086 mm thickness) and foil bags (0.112 mm thickness), and stored at room temperature and 4°C. The PPO, POD, PAL activities of dried-longan pulp were evaluated every month for 4 months.

2.4.2) Effect of different packaging materials on the quality changes of dried-longan pulp during storage time

The qualities in the terms of total phenolic content, color appearance, firmness, water activity, moisture content, titratable acidity and total soluble solid were

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evaluated. The data of these results were compared by using the statistical program of SPSS version 13.0.

(a) (b) (c)

Figure 2.6 The 3 differences packaging materials in this study;(a) LLDPE material; (b) OPP material; (c) Aluminum foil material

2.5 STATISTICAL ANALYSIS OF DATA

Statistical analyses of data used of SPSS version 13.0 were performed through an analysis of variance (ANOVA) and Duncan's multiple range test was used for comparing differences between means.

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