CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

1. Frozen green soya beans (AGS 292 and No.75 species) from Lanna Agro Industry Co., Ltd. (Chiang Mai, Thailand).
2. Skim milk (Mission, Swiss).
3. Sugar (Miltpol, Thailand).
4. Butter (Orchid, Thailand).
5. Mixture starters of *Streptococcus thermophilus*, *Lactobacilus acidophilus* and *Bifidobacterium bifidum* (FD-DVS ABT-5-Probio-Tec™, Christian Hansen, Denmark).
6. Kappa-carageenan (Nakarai Chemicals Ltd., Japan).
7. Carboxymethylcellulose (CMC) (Nakarai Chemicals Ltd., Japan).
8. Guar gum (Wendt-Chemie, Germany).

3.2 Chemical reagents

1. Sulfuric acid (Merck, Germany).
2. Sodium sulfate (Merck, Germany).
3. Copper sulfate (Merck, Germany).
4. Boric acid (Merck, Germany).
5. Diethyl ether (Merck, Germany).
6. Petroleum ether (Merck, Germany).
7. Ammonium solution 25% (Merck, Germany).
8. Ethyl alcohol 95% (Merck, Germany).
9. Sodium hydroxide (Merck, Germany).
10. Hydrochloric acid (Merck, Germany).
11. Phenolphthalein (Merck, Germany).

3.3 Chemical and media for culturing and enumeration of bacteria

1. Maximum Recovery Diluent; MRD (Oxoid, England).
2. M17 Broth (Oxoid, England).
3. Plate Count Agar; PCA (Merck, Germany).
4. Potato Dextrose Agar; PDA (Merck, Germany).
5. Lauryl Tryptose Broth (Himedia, India).
6. Lactose (Difco, USA).
7. Yeast extract (Merck, Germany).
8. Casamino acids (Bacto, France).
9. D (-)-fructose (Fluka, Switzerland).
10. Potassium dihydrogen phosphate (M&B, USA).
11. Tween 80 (Fluka, Switzerland).
12. Soytone (Bacto, France).
13. Peptone from casein (Merck, Germany).
14. Bromocresol green (Fisher Scientific, UK).
15. Agar (Himedia, India).

Note: The composition and preparation of these media were showed in Appendix D-3.

3.4 Equipment

1. An electric juice blender (Central, Thailand).
2. A blender (National, Thailand).
3. An ice cream maker model GC4000E (Simac Gelataio, France).
4. A freezer (Whirpool, Thailand).
5. A hot air oven model ULM 500 (Memmert, Germany).
6. An incubator model BE 400 (Memmert, Germany).
7. A water bath model WB 10 L1 (Memmert, Germany).
8. A pH meter model C830 (Consort, Belgium).
9. A chroma meter model CR-300 series (Minolta, Japan).
10. A hand refractometer (Atago, Japan).
11. Autopipettes (Pipetman, Germany).

12. A vortex model Ginie2 (Scientific, USA).
13. An autoclave (Gallenkamp, England).
14. A muffle furnance (Gallenkamp, England).
15. A 2100 Kjeltec distillation unit (Foss Tecator, Sweden).
16. A soxhlet extraction apparatus (Velp Scientifica, Thailand).
17. A DK 6 heating digester (Velp Scientifica, Thailand).

3.5 Research designs and methods

3.5.1 Analysis of green soya bean and green soya bean milk composition.

3.5.1.1 Preparation of green soya bean and green soya bean milk.

Washing green soya bean pods with cold water. Blanching the pods by boiling water for 2 min and cooling them. Separate green soya bean seeds from their skins. Blending green soya bean seeds until homogeneous using a blender and sampling for analyses.

Preparation for green soya bean milk. After separating the green soya bean seed from their skin, the green soya bean seeds were mixed with warm distilled water (at 60°C) at a ratio of 1:1 for the green soya bean seeds and warm distilled water, respectively. Extract the green soya been milk using a juicer and separate the milk from the solids. Take some samples of the green soya bean milk for analyses.

3.5.1.2 Analysis of green soya bean and green soya bean milk.

Physical analysis

Measurement for the sample colors using L*, a* and b* values by a Minolta colorimeter.

Chemical analysis

Fat of green soya bean was determined by a soxhlet extraction method (AOAC, 2000). Fat of green soya bean milk was measured by a Roese-Gottlieb method (AOAC,

2000). Protein was calculated by a protein factor of 6.38 after determination of nitrogen contents using a Kjeldahl method (AOAC, 2000). Fibers, carbohydrates, minerals based on total ash, moisture contents and total titratable acidities as % lactic acid were analysed according to AOAC methods (AOAC, 2000).

Total soluble solids were measured by a hand refractometer and pH values were assessed using a pH meter.

Microbiological analysis

Total viable microorganisms of the samples were evaluated by Total Plate Count. A pour plate method was applied together with PCA as a medium to grow the microorganisms. Plates were then incubated aerobically at 37°C for 48 h (Harrigan, 1998). The plates with numbers of microorganisms between 30-300 were used to estimate the numbers of microorganisms in the sample.

Statistical analysis

Collected data was analysed statistically using Analysis of Variance by applying Completely Randommized Design (CRD). A T-test was used to determine differences between treatment means ($p \leq 0.05$) following the procedure of Pongsirikool (2002).

3.5.2 Study the physical, chemical, microbiological and sensory characteristics of a prototype green soya bean yoghurt ice cream.

A product profile of a green soya bean yoghurt ice cream was prepared before developing the formula of green soya bean yoghurt ice cream by finding the ice cream characteristics that had high preferences from consumers. The method to develop the formula of the yoghurt ice cream was based on an ideal ratio profile. This method applied ratios of the sensory characteristics of a prototype product with those of an ideal product in determining the yoghurt ice cream product profile. To find the consumer preferences, several tests were conducted using a prototype green soya bean yoghurt ice cream. During the tests, 15 panelists were asked to determine the sensory characteristics of the prototype product on a horizontal line scale that had a value between 0-10. The panelists were also

asked to mark on the scale, the ideal preference value of the each sensory characteristic. The ratios between these two values were used to develop the product profile of green soya bean yoghurt ice cream (Wiriyajaree, 2002).

A prototype formula of green soya bean yoghurt ice cream was shown in Table 3.1.

Table 3.1 A prototype formula of green soya bean yoghurt ice cream.

Ingredients	Quantity (%) (w/v)
Main ingredients	
Green soya bean milk (green soya bean:water = 1:1)	86.67
Additional ingredients	
Sugar	6.93
Skim milk	4.33
Butter	1.73
κ-carrageenan	0.17
ABT-5 starter cultures	0.17
Total	**100**

3.5.2.1 Evaluation for the growth of starter cultures in green soya bean yoghurt ice cream formula.

ABT-5 starter cultures (FD-DVS ABT-5-Probio-Tec™, Christian Hansen, Denmark) were supplied in a freeze-dried form. The starter cultures contained a mixture culture of a thermophilic lactic culture of *Streptococcus thermophilus* and probiotic bacteria of *Lactobacillus acidophilus* LA-5 and *Bifidobacterium bifidum* BB-12.

The green soya bean yoghurt ice cream formula was shown in Table 3.1. The mixture was pasteurized at 85°C for 30 min, then cooled down with cool water and added with 0.17% (w/v) ABT-5 starter cultures. The inoculated milk mixture was incubated at 43°C for 6 h. The growth of the 3 starter cultures in the green soya bean yoghurt ice cream formula was assessed at the beginning and at the end of the fermentation period.

Measurement of *S. thermophilus* was carried out using a pour plate method on M17 agar and incubated aerobically at 37°C for 48 h. *L. acidophilus* (LA-5) and *B. bifidum* (BB-12) was assessed using a spread plate method on Homofermentative Heterofermentative Differential (HHD) agar and incubated anaerobically at 37°C for 72 h (Lourens-Hattingh and Viljoen, 2001).

Colonies characteristics and cells shapes of *S. thermophilus, L. acidophilus* and *B. bifidum* were also characterized based on their colony morphologies and under a microscope examination by using a Gram's staining method (Harrigan, 1998) that could be seen in Appendix D-5.

3.5.2.2 A production procedure for a prototype product of green soya bean yoghurt ice cream.

Green soya bean milk

Washing green soya bean pods with cold water. Blanching the pod by boiling water for 2 min and cooling them. Separate green soya bean seeds from their skins. Using a mixture ratio of green soya bean:warm distilled water (at 60°C) of 1:1, extracting the green soya bean milk by a juicer. Keeping the extracted milk for the next process.

Green soya bean yoghurt

Add 6.93% (w/v) sugar, 4.33% (w/v) skim milk, 1.73% (w/v) butter and 0.17% (w/v) kappa-carageenan into green soya bean milk from the previous process. Mixing properly all the ingredients at 70°C for 10 min. After that, blending the mixture with a blender at a hi-speed for 5 min and filling the mixture into a covered bottle. Pasteurizing the mixture at 85°C for 30 min. Cooling the mixture of green soya bean milk with cool water and adding with 0.17% (w/v) of ABT-5 starter cultures. Incubating the cultured green soya bean milk at 43°C for 6 h. Stop the fermentation process by cooling the green soya bean yoghurt in cool water. Keeping the green soya bean yoghurt in a refrigerator for 24 h for the next process.

Green soya bean yoghurt ice cream

Blending green soya bean yoghurt until homogeneous and processing it in an ice cream machine for 20 min. Transfering the green soya bean yoghurt ice cream into plastic cups (50 ml) and keeping the green soya bean yoghurt ice cream in a freezer for 24 h for physical, chemical and microbiological analyses. The processing diagram for the production of green soya bean yoghurt ice cream was displayed in Figure 3.1.

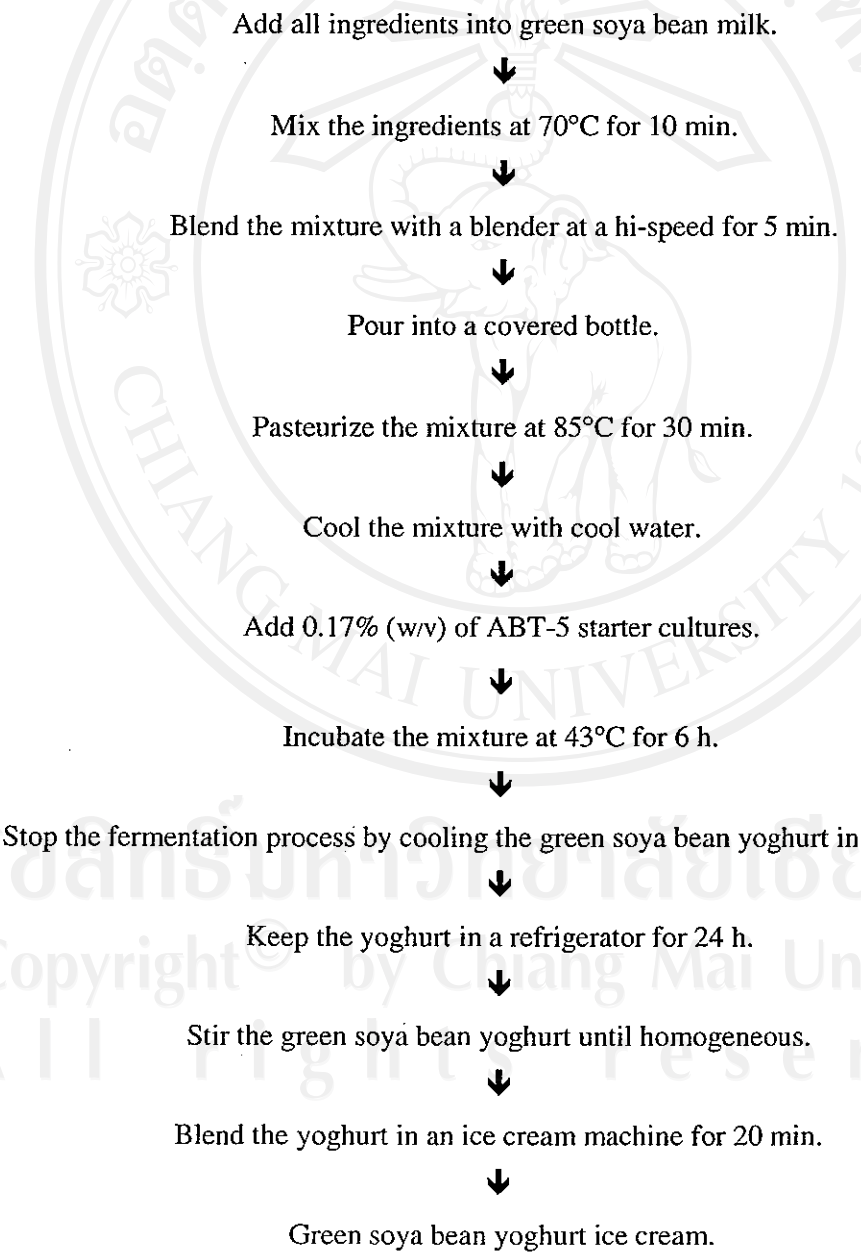

Figure 3.1 The production process of green soya bean yoghurt ice cream.

3.5.2.3 Survey product profiles.

Using a prototype product of green soya bean yoghurt ice cream, a product profile of green soya bean yoghurt ice creams was determined by a sensory analysis used 15 panelists. During the analysis, a ratio profile technique was employed to find out sensory parameters that needed to be improved (Wiriyajaree, 2002). In brief, the sensory test was carried out as followed. First, the panelists were given a cup of green soya bean yoghurt ice cream and a sensory questionnaire. In the questionnaire, the panelists were asked to mark on a horizontal line scale the sensory characteristic values of the prototype green soya bean yoghurt ice cream. Afterwards, the panelists should give their opinions regarding the best/ideal value for each sensory characteristic of the green soya bean yoghurt ice cream. The different values between the two points were used to develop an ideal product of green soya bean yoghurt ice cream.

3.5.2.4 Analysis of green soya bean yoghurt ice cream.

Physical analysis

Measurement of the ice cream colors based on L*, a* and b* values by a Minolta colorimeter. Measurement of the ice cream overrun by a procedure from Arbuckle (1996). Measurement of the ice cream melting rate by a method of Lee and White (1991).

Chemical analysis

Measurement of water contents and total titratable acidities as % lactic acid using procedures published by AOAC (2000).

Measurement of total soluble solids by a hand refractometer and pH values using a pH meter.

Microbiological analysis

Measurement of *S. thermophilus* was carried out using a pour plate method on M17 agar and incubated aerobically at 37°C for 48 h. *L. acidophilus* (LA-5) and *B. bifidum* (BB-12) was assessed using a spread plate method on Homofermentative Heterofermentative Differential (HHD) agar and incubated anaerobically at 37°C for 72 h (Lourens-Hattingh and Viljoen, 2001).

Sensory evaluation

Sensory analysis was carried out using an ideal ratio profile technique with 15 panelists following the procedure of Wiriyajaree (2002).

Statistical analysis

The sensory data were analysed statistically by using a T-test ($p \leq 0.05$) following the procedure of Pongsirikool (2002).

3.5.3 Development of green soya bean yoghurt ice cream formula.

3.5.3.1 Determination of green soya bean yoghurt ice cream ingredients that affect the quality of the yoghurt ice cream.

To find out the important green soya bean yoghurt ice cream ingredients that affect the quality of the final product, an experimental design of Plackett and Burman was used in this section. Since there were 8 ingredients (experimental factors) that needed to be studied, 3 dummy variables needed to be included in the design. From 11 experimental factors, the design was conducted with 12 experimental units. The complete Plackett and Burman design that was applied in this section could be seen in Table 3.2. In Table 3.3, it was shown the low and high levels of the 8 experimental factors that were studied in this research.

Table 3.2 The Plackett and Burman design for green soya bean yoghurt ice cream.

Experimental units	A	B	C	D	E	F	G	H	I	J	K
1	+	+	-	+	+	+	-	-	-	+	-
2	+	-	+	+	+	-	-	-	+	-	+
3	-	+	+	+	-	-	-	+	-	+	+
4	+	+	+	-	-	-	+	-	+	+	-
5	+	+	-	-	-	+	-	+	+	-	+
6	+	-	-	-	+	-	+	+	-	+	+
7	-	-	-	+	-	+	+	-	+	+	+
8	-	-	+	-	+	+	-	+	+	+	-
9	-	+	-	+	+	-	+	+	+	-	-
10	+	-	+	+	-	+	+	+	-	-	-
11	-	+	+	-	+	+	+	-	-	-	+
12	-	-	-	-	-	-	-	-	-	-	-

Note: A-H were the main experimental factors from the green soya bean yoghurt ice cream formula. I-K were dummy variables. (+) was a high level and (-) was a low level of the experimental factors.

The green soya bean yoghurt ice cream ingredients that were represented by alphabet letters were:

A was skim milk (%) (w/v).

B was butter (%) (w/v).

C was sugar (%) (w/v).

D was starter culture (%) (w/v).

E was carboxymethylcellulose CMC (%) (w/v).

F was κ-carrageenan (%) (w/v).

G was guar gum (%) (w/v).

H was green soya bean milk (%) (w/v).

Table 3.3 The level of experimental factors used in the Plackett and Burman design.

Experimental factors	Levels of the experimental factors	
	Low level (-)	High level (+)
A	5	10
B	2	6
C	8	16
D	0.2	0.4
E	0.1	0.4
F	0.1	0.4
G	0.1	0.4
H	40	80

For each of the experimental unit, the experimental factors were mixed properly and added with distilled water to become 1 l.

3.5.3.2 Finding the best level of the experimental factors that affect the quality of green soya bean yoghurt ice cream.

The experimental result from the section 3.5.3.1 was used in this section. From the statistical result of the section 3.5.3.1 (Chapter 4, section 4.3.1), it was shown that 2 ingredients of green soya bean yoghurt ice cream significantly affected the sensory quality of the final product. These ingredients were skim milk and sugar. In this section, these materials were studied futher in a factorial design experiment. The studied levels for skim milk and sugar were displayed in Table 3.4. For other ingredients that did not significantly affected the quality of green soya bean yoghurt ice cream, their levels could be seen in Table 3.5. The experiment was done in triplicate.

Table 3.4 The levels (%) (w/v) of skim milk and sugar of green soya bean yoghurt ice creams that were studied in a factorial design experiment.

Study factors	Low level (-)	Middle level (0)	High level (+)
Skim milk	8	10	12
Sugar	12	15	18

Table 3.5 The levels of green soya bean yoghurt ice creams ingredients that did not significantly affected the quality of the final product.

Compositions	Quantity (%)(w/v)
Butter	2
Starter culture	0.2
κ-carrageenan	0.1
CMC	0.4
Guar gum	0.4
Green soya bean milk	80
Distilled water	20

3.5.3.3 Analysis of green soya bean yoghurt ice cream.

Physical analysis

Measurement of the ice cream colors based on L*, a* and b* values by a Minolta colorimeter. Measurement of the ice cream overrun by a procedure from Arbuckle (1996). Measurement of the ice cream melting rate by a modified method from Lee and White (1991).

Chemical analysis

Measurement of water contents and total titratable acidities as % lactic acid using procedures published by AOAC (2000).

Measurement of total soluble solids by a hand refractometer and pH values using a pH meter.

Microbiological analysis

Measurement of *S. thermophilus* was carried out using a pour plate method on M17 agar and incubated aerobically at 37°C for 48 h. *L. acidophilus* (LA-5) and *B. bifidum* (BB-12) was assessed using a spread plate method on Homofermentative Heterofermentative Differential (HHD) agar and incubated anaerobically at 37°C for 72 h (Lourens-Hattingh and Viljoen, 2001).

Sensory evaluation

The sensory analysis of this section used a Balance Incomplete Block Design (BIB) following the procedure of Aooppadissakul (1994) and an ideal ratio profile technique following the procedure of Wiriyajaree (2002). There were 13 panelists that involved in the section 3.5.3.1 (a Plackett and Burman design) and 18 panelists that assessed in the section 3.5.3.2 (a factorial design experiment).

Statistical analysis

The physical, chemical and microbiological data were analysed statically using Analysis of Variance by applying a Plackett and Burman design and a factorial design in Completely Randommized Design (CRD) for the sections 3.5.3.1 and 3.5.3.2, respectively. The statistical analysis was carried out using a SPSS program version 10.0.1 for Windows (SPSS, Inc., Chicago, U.S.A.). Duncan's New Multiple Range Test (DMRT) was applied to determine differences between treatment means ($p \leq 0.05$) following the procedure of Pongsirikool (2002).

For the sensory data, the analysis result was statistically processed using Analysis of Variance by applying a BIB design. The statistical analysis was conducted using a SPSS program version 10.0.1 for Windows (SPSS, Inc., Chicago, U.S.A.). DMRT was applied to determine differences between treatment means ($p \leq 0.05$) following the procedure of Pongsirikool (2002).

3.5.4 Study the optimum time and temperature incubation for the production of green soya bean yoghurt ice cream.

3.5.4.1 Finding the optimum incubation condition for the production of green soya bean yoghurt ice cream.

The incubation time and temperature used during a fermentation process will affect the quality of the final product. One reason for this effect is because the acidity that is produced by starter cultures will be depended on the time and temperature of the incubation. An optimum time and temperature incubation will make a good quality product. In this section, the incubation factors of time and temperature to ferment green soya bean milk were studied in a factorial design experiment using 3 levels for each factor. The temperature (°C) and time (h) levels that were studied could be seen in Table 3.6. The composition of green soya bean yoghurt ice cream ingredients used in this section was displayed in Table 3.7.

Table 3.6 Different levels of temperature (°C) and time (h) incubation condition to ferment green soya bean milk.

Fermentation factors	Low level (-)	Middle level (0)	High level (+)
Temperature (°C)	40	43	46
Time (h)	4	6	8

Table 3.7 The composition of green soya bean yoghurt ice cream ingredients.

Ingredients	Quantity (%) (w/v)
Skim milk	9.02
Sugar	13.52
Butter	1.50
Starter culture	0.15
κ-carrageenan	0.08
CMC	0.30
Guar gum	0.30
Green soya bean milk	60.10
Distilled water	15.03
Total	100

3.5.4.2 Analysis of green soya bean yoghurt ice cream.

Physical analysis

Measurement of the ice cream colors based on L*, a* and b* values by a Minolta colorimeter. Measurement of the ice cream overrun by a procedure from Arbuckle (1996). Measurement of the ice cream melting rate by a modified method from Lee and White (1991).

Chemical analysis

Measurement of water contents and total titratable acidities as % lactic acid using procedures published by AOAC (2000).

Measurement of total soluble solids by a hand refractometer and pH values using a pH meter.

Microbiological analysis

Measurement of *S. thermophilus* was carried out using a pour plate method on M17 agar and incubated aerobically at 37°C for 48 h. *L. acidophilus* (LA-5) and *B. bifidum* (BB-12) was assessed using a spread plate method on Homofermentative Heterofermentative Differential (HHD) agar and incubated anaerobically at 37°C for 72 h (Lourens-Hattingh and Viljoen, 2001).

Sensory evaluation

The sensory analysis of this section was carried out using a Balance Incomplete Block Design (BIB) following the procedure of Aooppadissakul (1994) and an ideal ratio profile technique using 18 panelists following the procedure of Wiriyajaree (2002).

Statistical analysis

The physical, chemical and microbiological data were analysed statically using Analysis of Variance by applying a factorial design in CRD for this section. The statistical analysis was carried out using a SPSS program version 10.0.1 for Windows (SPSS, Inc., Chicago, U.S.A.). DMRT was applied to determine differences between treatment means ($p \leq 0.05$) following the procedure of Pongsirikool (2002).

For the sensory data, the analysis result was statistically processed using Analysis of Variance by applying a BIB design. The statistical analysis was conducted using a SPSS program version 10.0.1 for Windows (SPSS, Inc., Chicago, U.S.A.). DMRT was applied to determine differences between treatment means ($p \leq 0.05$) following the procedure of Pongsirikool (2002).

3.5.5 Study the shelf life of green soya bean yoghurt ice cream.

3.5.5.1 Production of green soya bean yoghurt ice cream.

In this section, the shelf life of green soya bean yoghurt ice cream during storage at -18°C was assessed. The green soya bean yoghurt ice cream formula used in this experiment followed the formula in the section 3.5.4 (Table 3.7). At the same time, one optimum condition to ferment the yoghurt ice cream formula was used according to the result of the section 3.5.4. After producing the green soya bean yoghurt ice cream, the product was filled into 50 ml plastic boxes and stored at -18°C for 4 months. During the storage period, representative samples were taken monthly and analysed for their physical, chemical, microbiological and sensory characteristics. The experiment was done in triplicate.

3.5.5.2 Analysis of green soya bean yoghurt ice cream.

Physical analysis

Measurement of the ice cream colors based on L*, a* and b* values by a Minolta colorimeter. Measurement of the ice cream overrun by a procedure from Arbuckle (1996). Measurement of the ice cream melting rate by a modified method from Lee and White (1991).

Chemical analysis

Fat of green soya bean yoghurt ice cream was measured by a Roese-Gottlieb method (AOAC, 2000). Protein was calculated by a protein factor of 6.38 after determination of nitrogen contents using a Kjeldahl method (AOAC, 2000). Fibers, carbohydrates, minerals based on total ash, moisture contents and total titratable acidities as % lactic acid were analysed according to AOAC methods (AOAC, 2000).

Total soluble solids were measured by a hand refractometer and pH values were assessed using a pH meter.

Microbiological analysis

Measurement of *S. thermophilus* was carried out using a pour plate method on M17 agar and incubated aerobically at 37°C for 48 h. *L. acidophilus* (LA-5) and *B. bifidum* (BB-12) was assessed using a spread plate method on Homofermentative Heterofermentative Differential (HHD) agar and incubated anaerobically at 37°C for 72 h (Lourens-Hattingh and Viljoen, 2001).

Measurement of total psychrotroph microorganisms was carried out using a pour plate method on Plate Count Agar and incubated aerobically at 5°C for 10 days (Al-Kadamany, 2003).

For yeasts and moulds, a method of Harrigan (1998) was used. Samples were plated by a pour plate method on Potato Dextrose Agar and incubated aerobically at 25°C for 5 days.

Coliform bacteria were monitored using a Most Probable Number (MPN) in Lauryl Tryptose Broth and incubated aerobically at 37°C for 24-48 h (Harrigan, 1998).

Sensory evaluation

Sensory analysis was carried out using an ideal ratio profile technique with 15 panelists following the procedure of Wiriyajaree (2002).

Statistical analysis

The physical, chemical and microbiological data from triplicate trials together with sensory evaluation result were analysed statistically using Analysis of Variance by applying CRD. The statistical analysis was conducted using a SPSS program version 10.0.1 for Windows (SPSS, Inc., Chicago, U.S.A.). DMRT was applied to determine differences between treatment means ($p \leq 0.05$) following the procedure of Pongsirikool (2002).