CHAPTER 3

Materials and methods

3.1 Instrument and chemicals

3.1.1 Instruments

Beaker heater: Gerhardt type EV 26, Germany

Blender: Osterizer, U.S.A.

Block Digestion Unit: Kjeldatherm, Gerhardt type TR, Germany

Boiling Sterilizer: Applied Medic Ltd., partnership, Bangkok, Thailand

Daisy^{II} incubator: model D200, Ankom technology, NY, USA.

Desiccator: Duran, Germany

Flask heater: Gerhardt type EV 26, Germany

Gas syringe 100 ml: Fortuna, Germany

Heat sealer: Audion Elektro, model 2355A, Netherlands

Hot plate and stirrer: Thermolyne Crimarec 2, lowa, U.S.A.

Kjeldahl: Gerhardt, Rapid Digestion System Type TT110, Rapid Distillation System Type 20, Germany

Magnetic stirrer: Ika model RO-10, Selangor, Malaysia

Muffle furnace: Heraeus type MR 260 E, Germany

Oven: Contherm Digital Series incubator, Lower Hutt, New Zealand

pH Meter: Mettler Toledo model CH-8603, Switzerland

Precision balance: Mettler Todedo model PB 3002, Urdorf, Switzerland

Refrigerator: Sharp model FC27 (-20ºC)

Soxhlet extractor: W. Krannich, Gottingen, Germany

Water purification System: Branstead model MP-11A, U.S.A.

3.1.2 Chemicals

Chemicals	Company	Grade
2-Ethoxyethanol	Merck	analytical
Acetone	Lab scan	analytical
Ammonium bicarbonate	Sigma	analytical
Boric acid	Merck	analytical
Calcium chloride dihydrate	Merck	analytical
Cetyl trimethyl ammonia bromide	Sigma	analytical
Cobalt (II) chloride hexahydrate	Merck	analytical
Cobalt (II) dihydrate	Merck	analytical
Copper (II) sulfate pentahydrate	Merck	analytical
Dichloromethane	Merck	analytical
di-Sodium ethylene diamine-tetraacetate	Merck	analytical
di-Sodium hydrogen orthophosphate	Merck	analytical
Hydrochloric acid	Merck	analytical
Iron (II) chloride hexahydrate	Merck	analytical
Magnesium sulfate	Merck	analytical
Magnesium Sulfate Heptahydrate	Merck	analytical
Manganese (II) chloride tetrahydrate	Merck	analytical
n-Hexane	Merck	analytical
Potassium dihydrogen phosphate	Merck	analytical
Sodium bicarbonate	Sigma	analytical
Sodium borate decahydrate	Merck	analytical
Sodium carbonate	Merck	analytical
Sodium chloride	Merck	analytical
Sodium hydroxide	Merck	analytical
Sodium hydrogen carbonate	Merck	analytical
Sodium sulfide nonahydrate	Sigma	analytical
Sulfuric acid	Merck	analytical

Chemicals	Company	Grade
Urea	Merck	analytical

3.2 Experimental site

This study were carried out at the experimental farm of the Department of Animal and Aquatic Science, Faculty of Agriculture, Chiang Mai University, Chiang Mai Province, Thailand (latitude 18°47'N and longitude 98°59'E).

The chemical analyses were conducted at the Department of Animal and Aquatic Science, Chiang Mai University, Thailand.

Figure 3.1 The field of this experiment

3.3 Forage management and harvest

Pangola, Napier, and Ruzi grass were harvested from the same location at the same regrowth age cutting (45 days). Fertilizer was applied at 20 kg (namely 46-0-0) per rai after cutted all types forage. For fresh pangola, napier and ruzi grass, their fields were conducted random sampling into different plots that maturity could be controlled by cutting at 45 days regrowth duration. For pangola hay, pangola grass was harvested and sun-dried on the field for 2-3 days, backscatter 2 times a day, morning and evening, so the grass gets sun throughout the pile. The hay for 3 days can check the dryness by using a fingernail scraping grass stems. If the surface of the grass stems out of that the

grass is still moisture. For pangola silage, pangola grass was used for 2 treatments, pangola silage without molasses and pangola silage with molasses 5 %. After which 5 kg sugarcane molasses per 100 kg fresh pangola was added in pangola silage with molasses. The materials were then homogenized and filled in plastic barrels each 10 kilograms, compacted, sealed and ensiled at room temperature for a minimum of 21 days. Each barrel was weighed before and after ensiling to determine the DM loss.

$$\text{DM loss (\%)} = \left\{ \frac{(\text{DM x weight}/100)_{\text{before ensiling}} - (\text{DM x weight}/100)_{\text{after ensiling}}}{(\text{DM x weight}/100)_{\text{before ensiling}}} \right\}$$

The all forages were used for produce six treatments, as follows:

Treatment 1	Fresh Napier (FN)
Treatment 2	Fresh Ruzi (FR)
Treatment 3	Fresh Pangola (FP)
Treatment 4	Pangola hay (PH)
Treatment 5	Pangola silage (PS)
Treatment 6	Pangola silage added 5 % molasses (PSM)

3.4 Animals

Four ruminally fistulated cattles (thai native cattles) fed fresh pangola *ad libitum* consumption and 2 kg concentrate feed. Fresh water was available continuously consumed during the whole experiment.

Rumen fluid was collected from four ruminally fistulated cattles. Rumen fluid was percolated through two layers of gauze into a warm bottle in a container (39°C), sealed immediately and transported to the laboratory.

Figure 3.2 Rumen fluid collected from fistulated cows that was kept under 39°C condition

3.5 The experiments

3.5.1 Experiment 1 Analyses of nutrient composition of fresh and preserved pangola grass at 45 days of regrowth stage cutting

3.5.1.1 Chemical composition analysis

Sample preparation

Feed samples were dried at 60°C in a forced-air oven overnight. After dried, samples were grounded to pass 1 mm screen. Ground samples were stored individually in airtight containers until analysis.

Six forage treatments were chemical analysis using proximate analysis (AOAC, 2006): dry matter (DM), ash, crude protein (CP) and ether extract (EE) and detergent analysis (Goering and Van Soest, 1970): crude fiber (CF), neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL). In most laboratories, some data is used to predict total digestible nutrients (TDN) of forages

For fresh grass:

TDN (% of DM) = -21.7656+1.4284(%CP) + 1.0277(%NFE)
+ 1.2321(%EE) + 0.4867(%CF)

For hay:

TDN (% of DM) = -17.2649+1.2120(%CP) + 0.8352(%NFE)
+ 2.4637(%EE) + 0.4475(%CF)

For silage:

TDN (% of DM) = -21.9391+1.0538(%CP) + 0.9736(%NFE)
+ 3.0016(%EE) + 0.4590 (%CF)

3.5.1.2 Silage characteristics

At opening, silages were checked by sensory evaluation (organoleptic quality) and each barrel was sampled for determination of pH. Organoleptic test was evaluated by use the grading criteria to assess the quality silage: color, odorless and texture and pH (Animal Nutrition Division, 2004) (Details in appendix 1). For determination of pH; placed both pangola silage 50 grams chopped into pieces 2-3 cm length and distillate water 200 mL into a blender jar and blended 30 seconds after that filtered thru two layers cheese cloths and then reading data on pH meter. The concentrations of lactic, acetic and butyric acids were analyzed by distillation procedures as described by Zimmer (1966). Fermentation quality of the silages was assessed with the scheme based on the concentrations of acetic acid, butyric acid and lactic acid.

3.5.1.3 *In vitro* gas production measurement for treatments

Methods

In vitro gas production was determined according to Menke and Steingass (1988). Samples of the air-dry feedstuffs were accurately weighed into 100 ml capacity glass syringes fitted with plungers with 230 mg sample/incubation/syringe in a single run. Syringes were filled with 30 mL mixed rumen fluid and buffer solution as described by Menke and Steingass (1988). Triplicates of standard hay and a standard

concentrate that can be obtained from the Institute of Animal Nutrition, Hohenheim University, 70593 Stuttgart, Germany, were included. The syringes were placed in a rotor inside the incubator (39°C) with about one rotation per min. Readings were made at 0, 3, 6, 9, 12, 24, 48, 72, 84 and 96 h of incubation. At the end of the incubation period, data were calculated by using equation.

Figure 3.3 *In vitro* gas production samples preparation

Figure 3.4 Placed sample syringes in a rotated incubator

Preparation of Artificial Saliva

	Volume (ml)			
Artificial Saliva – final volume	**500**	**1000**	**1500**	**2000**
Distilled water	237.5	475.0	712.5	950.0
Micromineral solution	120.0	240.0	360.0	480.0
Buffer solution	120.0	240.0	360.0	480.0
Micromineral solution	0.06	0.12	0.18	0.24
Resazurin	0.61	1.22	1.83	2.44
Reducing Solution				
Distilled water	23.8	47.5	71.3	95.0
1M NaOH	1.0	2.0	3.0	4.0
Na2S9·H2O (mg)	168	336	504	672

Source : Adapted from Menke and Steingass (1988)

Calculation

$$Gb\ (mL/230\ mg\ DM, h) = \frac{(V_h - V_0 - Gbo)*((230*(FH+Fc)/2)}{W}$$

V_h = volume gas at time h

V_0 = volume starter before incubation

Gbo = volume gas of blank sample

FH = 44.16/(GbH-Gbo) = Roughage correction

Fc = 62.6/(Gbc-Gbo) = Concentrate correction

W = weight of sample

Organic matter digestibility (OMD), metabolisable energy (ME)

OMD (%) = 14.88+ 0.889 GP + 0.45 CP + 0.065 Ash

Where; GP = 24 h net gas production (ml/200mg DM)

CP = Crude protein (g/kg DM)

Ash = Ash content (g/kg DM)

ME (MJ/kg DM) = 2.20+0.136 GP + 0.0574 CP

Where; GP = 24 h net gas production (ml/200mg DM)

CP = Crude protein (g/kg DM)

3.5.2 Experiment 2 *In vitro* digestibility of treatments using DAISY^{II} incubator technique

Methods

The Daisy^{II} *in vitro* incubation procedure was performed according to the ANKOM technology bulletin. *In vitro* digestion method had several steps: 0.25 g substrate in quadplicate for each of the four incubation replicates preparing in filter bag (F57); buffer solutions (solution A:B; 5:1), rumen inoculum for 24 and 48 h of incubation. The Daisy^{II} *in vitro* incubator was pre-set at 39°C with a rolling speed of 49 seconds per round. CO_2 was pumped into the incubator for 30 secs before the sample was inserted. Each flask has an inset plastic divider that acts to force incubated in vitro bags under the surface of the fluid on each rotation. The 24 incubation bags were then placed in the jar, 12 on each side of the internal divider, and the bottle was purged with CO_2 prior to adding the rumen inoculum and again before attaching the top and placing the jar in the incubation box. After 24 and 48 h of incubation, jars were removed from the incubation box and bags were removed and washed in cold tap water until the wash water was clear.

Analytical procedures

The incubated nylon bag forage samples were analyzed for DM according to AOAC (2006). The NDF residues in forages and bag residues were determined using reagents and methods as described by Van Soest *et al*. (1991) with exceptions. These were that NDF was calculated as the residue in the *in vitro* bags after 60 min of immersion in boiling ND with sodium sulfite.

Figure 3.5 Daisy^{II} incubator (ANKOM technology)

Calculation

$$NDFD\ (\%\ DM) = 100 \times [(W2 \times \%NDF_{Feed}) - (W3 - (W1 \times C1))]/(W2 \times \%DM_{Feed})$$

$$IVDMD\ (\%DM) = 100 - \frac{[(W3 - (W1 \times C1)) \times 100]}{(W2 \times \%\ DM_{Feed})}$$

Where: W1 = Bag tare weight

W2 = weight of sample

W3 = final weight (Filter bag + sample)

NDF_{Feed} = NDF contain in Feed (%DM)

DM_{Feed} = dry matter contain in feed

C1 = correction of factor (blank filter bag)

3.5.3 Experiment 3 *In sacco* digestibility by nylon bag technique

Methods

DMD were determined by incubating 2.4–2.5 g of dry sample in nylon bags (41 m pore size and 6.5 cm × 14 cm dimension) in four rumen fistulated thai native cattles. Cattles were fed ad libitum pangola grass and 2 kg concentrate feed once daily. Steers were kept indoors and water was offered ad libitum. Bags were incubated and withdrawn sequentially after 8, 12, 24, 48, 72 and 96 h. After removal, the bags were washed in cold water for 25 min to inhibit further microbial activity, using a household washing machine and dried for 48 h at 60°C to determine the residual dry matter from which dry matter disappearance (DM_d) were calculated.

Calculation

$$\text{DM undegradability (\%)} = \frac{DM_{after} \times 100}{Weight_{before}}$$

$$\text{DM degradability} = 100 - \left[\frac{DM_{after} \times 100}{Weight_{before}}\right]$$

Where: DM_{after} = Dry matter of feed after incubation

$Weight_{before}$ = Weight of feed before incubation

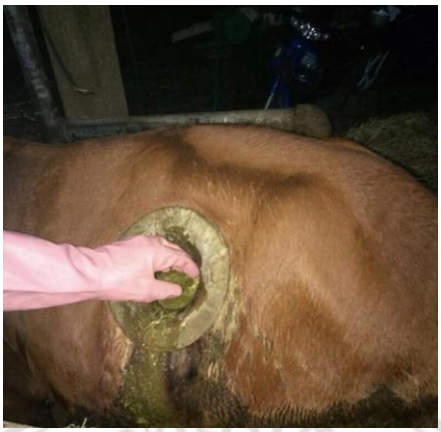

Figure 3.6 Placed nylon bags in fistulated cattle

Analysis

The data were fitted to the model of Ørskov and McDonald (1979) by NEWAY computer package program to an exponential equation $Y = a + b\ (1 - e^{-ct})$. The effective degradability (ED) was calculated using the formula $ED = a + [bc/(c + k)]$ (Dhanoa, 1988) where a, b and c are as described above and k is the rumen outflow rate, assumed to be 0.02, 0.05 and 0.08/h (Ørskov *et al*., 1988). Potential degradability (PD) (g/kg) was calculated as a + b.

3.6 Statistical analysis

The all stages experiment data were analyzed for significant differences among various treatments by analysis of variance (ANOVA) using Completely Randomized Design (CRD) and the means were compared by Duncan's Multiple Range Test (DMRT) using program SPSS 20.0.