III. MATERIALS AND METHODS

III. 1 Chemicals and Reagents

All the chemicals used were analytical grade except for cell culture or HPLC grade as otherwise stated and the lists are according to the suppliers.

Sigma Chemicals Company, U.S.A.

Ficoll-hypaque (Histopaque Density 1.077), trypan blue dye, phytohemagglutinin (PHA-P) , poke weed mitogen (PWM) , concanavalin A (Con A) , standard (+) - α - tocopherol

Merck, West Germany

Hydrochloric acid , sodium chloride, sodium hydrogen bicarbonate, sodium (+) - L - ascorbate , Potassium hydroxide

J.T. Baker

Absolute ethanol , methanol(HPLC grade) , n-hexane (HPLC grade)

Others

Fetal calf serum (Seromed, Germany), Gentamicin (Roussel Laboratories, England), Heparin (Leo, Denmark), MEM, minimum essential medium with Earle's salt and L-glutamin (Gibco, U.S.A.), RPMI-1640 medium with L-glutamin (Seromed, Germany), Penicilin G sodium (M&H Manufacturing, Thailand), Streptomycin sulfate (M&H Manufacturing, Thailand), [methyl-^{3}H] thymidine specific activity 185 G Bq/mmol (Amersham), Tocopheryl acetate, feed grade (Rovithai Ltd.)

III.2 Experimental Instruments

- Analytical balance
- Biohazard laminar flow (BLF - 120, Thailand)
- Carbondioxide incubator (Forma Scientific, U.S.A.)
- Water bath (Rost Frei, Germany)
- pH meter

- Refrigerator (-20°C)
- Filter holders with funnel (U.S.A.)
- Pasteur pipette (pyrex, U.S.A.)
- Membrane Filter 47 mm. 0.2 μm (Gelman Sciences, U.S.A.)
- Cell harvester
- Micro Beta counter (Trilux, Finland)
- Autoclaving pot
- 96 well round bottom culture plate (corning, U.S.A.)
- 96well flat bottom culture plate (Corning, U.S.A.)
- Multichannel pipet
- Pipet tip 1,000 , 50 - 200 μl
- High Performance Liquid Chromatography (Shimadzu, Japan)
- HPLC Inertsil ODS - 3 column (Gl Sciences, Japan)
- HPLC guard column Inertsil ODS - 3 (Gl Sciences, Japan)
- Syringe 100 μl (SGE, Australia)
- Filter, pore size 0.45 μm (Sartorius, Germany)
- Microscope (Olympus, Japan)
- Invers microscope (Olympus, Japan)
- UV - Visible spectro photometer (Beckman, U.S.A.)
- Filter holder (Milipore,)

III. 3 Methods

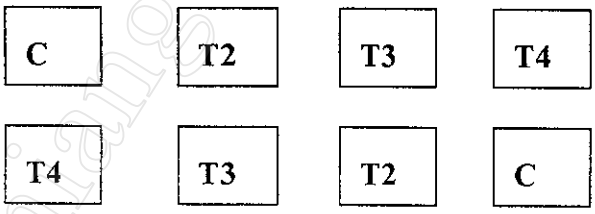
The study was divided into two experiments; Experiment 1 was carried out to find out the effects of vitamin E supplemental diets on pig humoral immune response in which Aujeszky's disease (AD) antibody titer after vaccination is used as a monitor. Experiment 2 was carried out to investigate the effects of vitamin E supplemental diets on pig cellular immune response in which *in vitro* lymphocyte proliferation activated by mitogens is used as indicator.

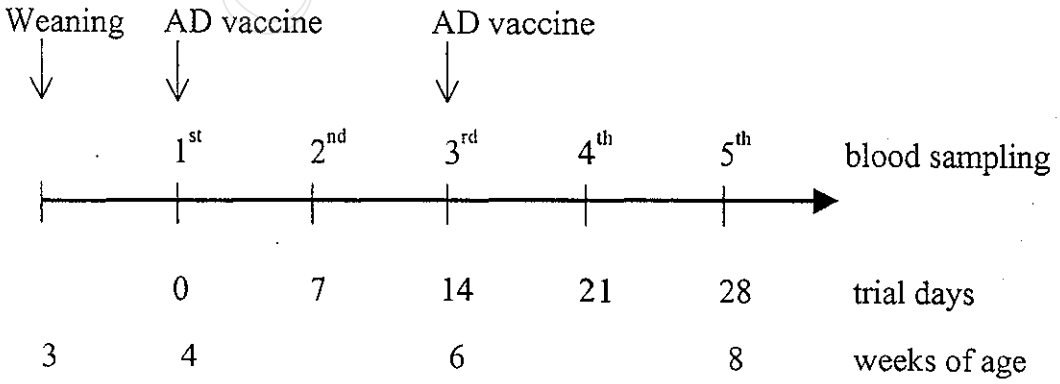
III. 3.1 Experimental design

Experiment 1 : This experiment was carried out at the Vorapong farm, a commercial pig production farm in Mearim district, Chiangmai.

80 Landrace x Large white x Duroc crossbred pigs approximately 3 weeks old were randomly penned ten pigs to each pen. A completely randomized design was applied to the experiment. Four dietary vitamin E treatments ; 0, 50, 100, and 200 mg/kg, were randomly allocated to two pens in each. All pigs were healthy, castrated males of approximately the same weight. The week before commencing the trial, the pigs were allowed to adapt to the experimental conditions. Feed and water were given *ad lib.* throughout the experiment. On the first week, all pigs were given AD vaccine. Following as the routine farm's vaccine program, hog cholera, boosted AD, and boosted hog cholera vaccine were given at one-week intervals respectively. Only AD antibody titers were used to assess the effect of vitamin E treatments on humoral immune response. Pig sera were aseptically collected before vaccination and revaccination five times and stored at -20 °C until antibody titer testing.

Model :Experiment 1

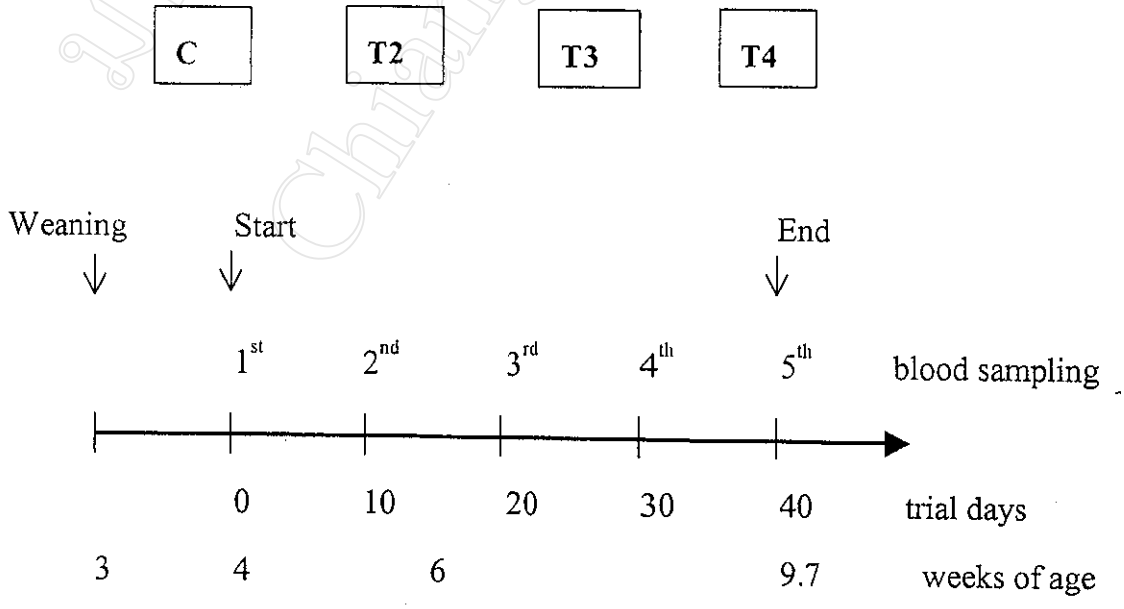




Experiment 2 : This experiment was conducted at the animal experimental units of the Animal Science Department, Faculty of Agriculture, Chiangmai University.

24 Landrace x Large white x Duroc crossbred weaned pigs approximately 3 weeks old were randomly grouped with an equal number of females and castrated males and housed six a pen. A completely randomized block design was performed. Four dietary vitamin E treatments (0, 50, 100, and 200 mg/kg) were randomly assigned to four pens. Feed and water were available *ad lib.* during the 42 day trial. One week prior to initiating the experiment was allow for the pigs to acclimatize. Hog cholera and foot and mouth disease vaccines were given according to the farm's program. Blood samples were obtained from jugular veins into sterile heparinized tubes and subjected to *in vitro* lymphocyte blastogenic test. The blood collections were divided into three groups of 8 pigs spanning the treatments because of the lab. facilities. The tests were carried out at five intervals of ten days throughout the experiment.

Model : Experiment 2



III. 3.2 Feed preparation

Experimental ration was a practical feed used long term on the farm and approved to meet nutrient requirements for weaned pigs in order to minimize other immuno-nutritional effects. The ration was prepared as a single formular (see appendix table1) and dry mash feed. After being properly mixed, one fourth of ration was removed as control dietary treatment and the remaining feed was supplemented and remixed with vitamin E to reach three concentrations; 50, 100 or 200 mg per kg diet. The lower concentration was drawn out prior to achieving the higher one respectively. The ration was packed tightly and placed in dry, dark room. The dietary treatments were sampled to determine the precise existing total vitamin E, from natural and supplemented source.

III. 3.3 Determination of AD antibody titer

III. 3.3.1 Serum collection

5 ml. of blood were drawn from jugular veins into non-anticoaggulant sterile tubes. The blood was allowed to clot. Serum was obtained by centrifugation, transferred to aliquots and frozen at -20 °C until the determinations of AD antibody titer and serum α-tocopherol concentration had been carried out.

III.3.3.2 Microtitration serum-virus neutralization (SN) test for AD titer

The test was modified from Hill *et al.* (1977) and set up as a standardized method used in the laboratory of Virology Section at the National Institue of Animal Health, Department of Livestock Development, Bangkok, Thailand.

Principle : Serum containing specific antibodies could neutralize specific viral infection or protect culture of target cells from cytophatic effect (CPE) occurring. Antibody titer was performed by varying serum dilution and the highest dilution still existing complete viral inhibition was AD titer.

Reagents

1. Inactivated test sera, inactivate at 56 $^{\circ}$C for 30 minutes
2. Cell suspension of pig - kidney (PK - 15) cell line ; well grown cells in log phase of growth about 3 days subculture ; concentration approximately 400,000 cells/ml, an appropriate concentration of cells to obtain a complete monolayer in 48 hours.
3. Cell culture medium (MEM), pH 7.2 - 7.4 with antibiotic supplement
4. Test virus, the shop strain of Pseudo-rabies virus, preparation of 50 percent tissue culture infective dose ($TCID_{50}$).

Procedure

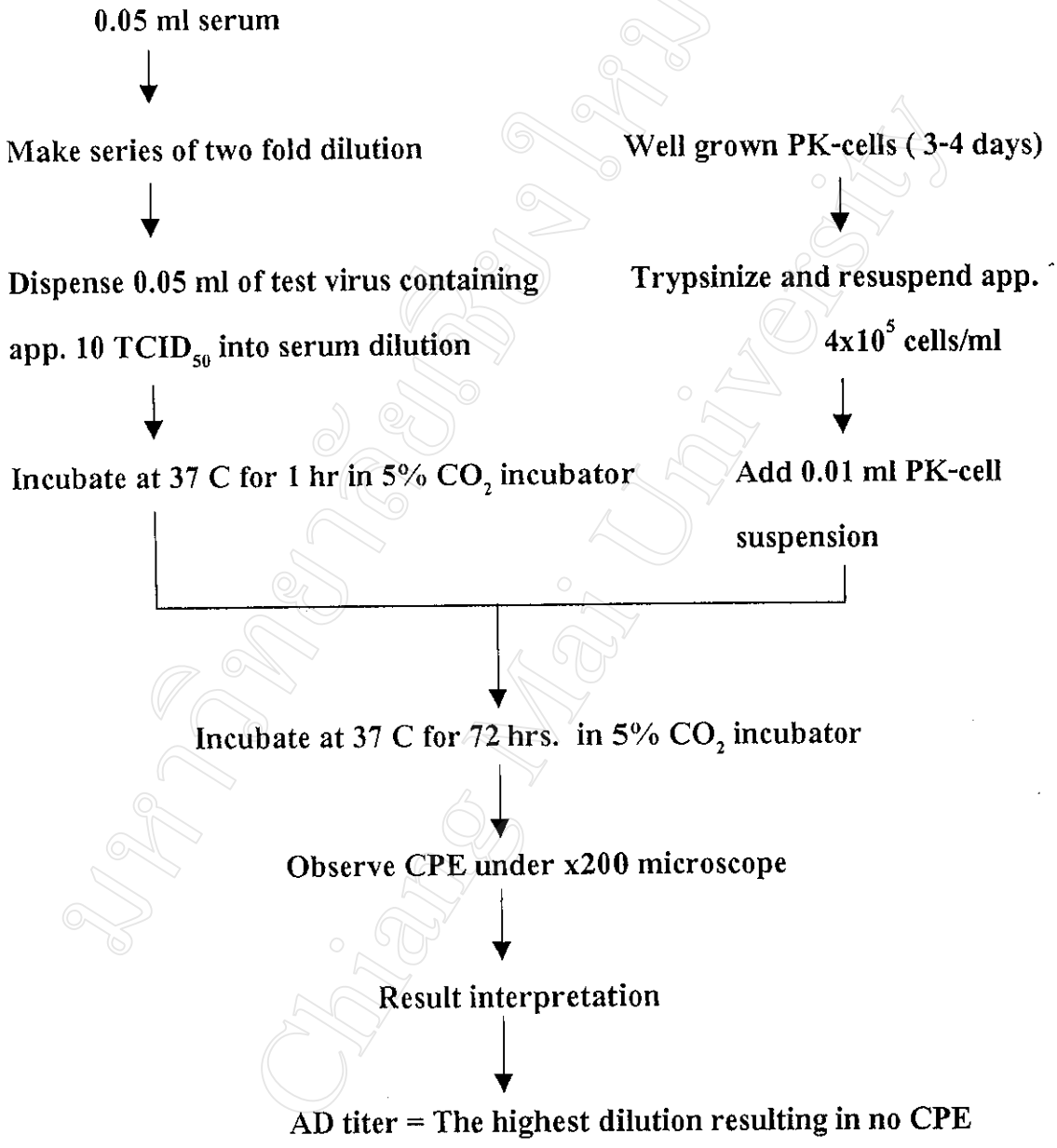
1. 50 µl of inactivated test sera and 50 µl of MEM (diluent) were pipetted into the first column well of a 96 well flat bottomed plate. All tests were duplicated. Serial two fold dilutions of test sera were made across plate column previously containing 50 µl of diluent.
2. 50 µl of test virus containing approximately 10 $TCID_{50}$ were dispensed into wells containing diluted sera, except serum control wells which received an equal volume of diluent instead.
3. After mixing thoroughly, the test plate was incubated at 37 $^{\circ}$C in 5% CO_2 incubator for 1 hour.
4. 100 µl of PK - 15 cell suspension were allocated to each well including control wells.
5. The tests were incubated at 37 $^{\circ}$C for 72 hours in 5% CO_2 incubator
6. A back titration of the virus used in each day's test which is necessary for quality control should be accompanied. A serial tenfold dilution; undiluted, 10^{-1}, 10^{-2}, and 10^{-3} of the test virus was made and 50 µl of viral suspension were dispensed into 8 wells each dilution.
7. 100 µl of PK-15 cell suspension was added to each well and treated in the same way as the test plate previously mentioned.

Result interpretation

The highest dilution of serum, in duplicate, resulting in complete inhibition of viral invasion causing cell damage or no (CPE) is considered the antibody titer to AD.

The control wells should contain no CPE and the results of back titrations should correspond to the prepared $TCID_{50}$

Determination of AD titer by SN-test



Modified from Hill *et al.*, (1977)

III. 3.4 Determination of lymphocyte proliferation

Principle : The ability of lymphocyte proliferating response to mitogens; PHA and Con A, a major T - cell activator and PWM, a major B - cell activator was determined by using radio technique; the radio ^{3}H-thymidine was incorporated into newly synthesized DNA resulting from cell transformation.

Reagents

1. Heparin 1,000 unit/ml in PBS
2. RPMI-1640 medium, pH 7.2-7.4 with antibiotic and Na_2HCO_3 2.0 g/liter
3. Histopaque
4. Fetal calf serum, inactivated at 56 °C for 30 minutes
5. 0.25% trypan blue in saline
6. PHA - P, 20 μg/ml
7. PWM, 10 μg/ml
8. Con A, 24 μg/ml
9. [methyl - ^{3}H] thymidine 4 μCi/ml
10. Scintillation fluid

III. 3.4.1 Blood collection and peripheral blood mononuclear cells (PBMCs) separation

Principle : PBMCs were separated by Ficoll-hypaque gradient centrifugation modified from Lessard *et al.*, (1991). All steps were performed aseptically.

Procedure

1. 10 ml of blood sample were collected from jugular veins into a sterile tube containing 200 units of heparin as anticoaggulant.
2. Plasma was obtained by centrifugation at 1,500 rpm for 10 minutes into aliquots and keep at -20 °C to determine plasma α - tocopherol
3. Packed blood were resuspended cells and made up to 10 ml with RPMI-1640, serum free medium (SFM)

4. 4 ml of Histopaque were layered under diluted blood gently to obtain two separated layers and the tube were capped tightly.
5. The tube was centrifuged at 2,500 rpm. for 30 minutes at room temperature.
6. The interface smeary white brand containing PBMCs was gently aspirated into into a tube containing 10 ml SFM
7. PBMCs were washed twice by resuspending and centrifugation with SFM
8. Cells were counted and adjusted to the concentration of 2.5 x 10^6 cells/ml in 10% FCS supplemented RPMI medium by trypan blue exclusion test,
9. The cell suspension was kept in 4 °C ice bath to maintain good viability which should be higher than 98%.

III. 3.4.2 Lymphocyte proliferation culture

Procedure

1. 0.1 ml of cell suspension were dispensed in a 96 well round bottomed culture plate, resulting in 250,000 cells per well.
2. The cultures were added 0.1 ml of mitogents ; with 20 μg/ml PHA, 10 μl/ml PWM, and 24 μg/ml ConA, previously titrated, in triplicate. The control cultures received an equal volume of SFM instead of mitogen.
3. The cultures were incubated at 37 °C for 48 hours in the 5% CO_2 incubator; cell clumps could be seen clearly overnight.
4. The cultures were pulsed with 0.05 ml (0.2 μCi) ^{3}H-thymidine per culture and incubated over 18 hours.
5. The culture was frozen and then thawn to harvest cells on a filter math, a glass fiber paper, by automatic cell harvestor.
6. The filter math was dried overnight at 40 °C.
7. The filter math was sealed along with proper volume of scintillant in a plastic bag.
8. The radio count was executed by Micro Beta counter.

The data were obtained in average counts per minute (cpm).The result was expressed in Δ cpm, the mean cpm of activated culture minus the mean of control one.

PBMCs separation

10 ml heparinized blood

↓

Collect plasma and resuspend blood cells in RPMI-1640(SFM)

↓

Under layer with 4 ml Histopaque (D = 1.077g/ml)

↓

Centrifuge at 2500 rpm , RT. for 30 min.

↓

Collect interface band of PBMCs

↓

Wash x2 by centrifugation with SFM

↓

Count and adjust to $2.5x10^6$ cells/ml

↓

By trypan blue exclusion test (viability >98%)

Modified from Lessard *et al.*, (1991)

Lymphocyte proliferation test

Dispense 0.1 ml of PBMCs suspension in round bottomed plate

↓

Add 0.1 ml mitogen (in triplicate)

PHA; 20 μg/ml, Con A ; 24μ g/ml

PWM ; 10 μg/ml, SFM for control culture

↓

Incubate at 37 $^{\circ}$C for 48 hrs. in 5% CO_2 incubator

↓

Pulse with 0.2 μCi ^{3}H-thymidine / well

↓

Incubate for 18 hrs. more

↓

Harvest culture on glass fiber paper

↓

Dry filter match

↓

Count radioactive by Beta scintillation counter

Modified from Lessard *et al.*, (1991)

III. 3.5 Determination of plasma or serum α-tocopherol

Principle: This method was slightly modified from Hess *et al.*, (1990) and Ortiz *et al.*, (1991).

Reagent

1. Test serum or plasma
2. Distilled water
3. Ethanol
4. Pure methanol (HPLC grade)
5. n - Hexane (HPLC grade)
6. Standard (+) -α-tocopherol mixed isomers.

Procedure

1. 0.25 ml serum and 0.25 ml distilled water were pipetted into a screw cap tube with a polyethylene stopper.
2. 0.5 ml ethanol were added and the tube was mixed well for 10 seconds.
3. 1.0 ml n-hexane was dispensed in the mixture and the tube was closed rapidly.
4. The tube was shaken at 300 rpm speed for 20 minutes.
5. Then was centrifuged at 2,500 rpm for 10 minutes.
6. 0.4 ml. n-hexane layer (upper) was transfered and the supernatant was dried under nitrogen stream in a light protected tube.
7. The residue was dissolved in 1.0 ml pure methanol and then the extracted solution was applied to the HPLC method.

HPLC condition for α-tocopherol

Column : stainless steel, length 15.0 cm, inner diameter 4.6 mm.

Stationary phase : inertsil ODS - 3.

Mobile phase : pure methanol.

Column temperature : 28 ^{o}C.

Flow rate : 1.5 ml/minute.

Pressure : approximate 57 bar.

Injection volume : 20 μl.

Detector : fluorescence.

Detection wavelengths : excitation 293 nm, emission 328 nm.

Retention time :7.8 minutes.

Calculation : Quantification was automatically executed by the external standard method based on area counts.

Extraction of vitamin E in plasma or serum

0.25 ml serum or plasma + 0.25 ml dist. water

+ 0.5 ml ethanol

↓

Mix well for 10 sec.

↓

Add 1.0 ml n-hexane

↓

Shake at 300 rpm for 10 min.

↓

Transfer 0.4 ml n-hexane layer into a new tube

↓

Dry the supernatant under N_2 stream

↓

Dissolve the residue in 1.0 ml pure methanol

↓

HPLC injection

Modified from Hess *et al.*, (1990) and Ortiz *et al.*, (1991)

III.3.6 Determination of total vitamin E in feed

Principle : The method was modified from A.O.A.C. (1990). Total vitamin E in feed was extracted into absolute ethanol, saponified in alkaline mixture, and reextracted with n - hexane and HPLC method was used for quantification.

Reagent

1. 10 grams of finely grained feed.
2. Absolute ethanol
3. 10% (w/v) sodium ascorbate
4. 50% (w/v) potassium hydroxide
5. n-Hexane (HPLC grade)
6. Pure methanol (HPLC grade)
7. Sodium sulphate anhydrous.

Procedure

1. 10 g feed sample were weighed into a thimber.
2. The thimber was placed in a round bottomed flask.
3. 100 ml absolute ethanol were added and the solvent level was marked.
4. The flask was applied to a condenser on a hot water bath (at 80 $^{\circ}$C) and refluxed over 8 hours.
5. The thimber was rinsed with ethanol into the flask until the solvent mark was reached then removed.
6. 20 ml extracted solution were transferred into a new flask.
7. 1 ml 10% sodium ascorbate and 5 ml 50% KOH were added.
8. The mixture was refluxed in a hot water bath (at 80 $^{\circ}$C) for 15 minutes.
9. The cooled mixture was transferred and the flask was rinsed with 10 ml distilled water into a separated funnel.

10. The mixture was extracted three times by vigorous shaking with 10 ml n-hexane.
11. The hexane phase (upper) was collected and washed twice with 100 ml. distilled water.
12. Any moisture in solvent was removed by adding sodium sulphate anhydrous.
13. 1 ml. clear supernatant was transferred and dried under nitrogen stream.
14. The residue was dissolved in 1 ml methanol
15. Total α-tocopherol was quantitated by HPLC method.

III.3.7 Production performance recording

Initial and final weights were recorded to calculate an average daily gain. Total feed intake per pen was recorded throughout the experiment. Feed conversion ratio represents the whole group of pigs.

Extraction of vitamin E in ration

Weigh 10 g finely grind feed into a thimber

↓

Place the thimber in a round bottomed flask

and add 100 ml absolute ethanol

↓

Reflux on hot water bath (80 °C) for 8 hrs

↓

Make up ethanol to initial volume

↓

Pipette 20 ml extracted solution into a new flask

↓

Add 1 ml 10 % sodium ascorbate and 5 ml 5 % KOH

and reflux for 15 min.

↓

Transfer cooled mixture into separated funnel

and rinse with 10 ml dist. water

↓

Remove moisture by adding sodium sulphate anhydrous

↓

Dry 1 ml supernatant under N_2 stream

↓

Dissolve the residue in 1 ml methanol

↓

HPLC injection

Modified from A.O.A.C. (1990)