11.8 *In vitro* determination of degree of protection in bypass fat supplement

Equipment

1. Stoppered ground glass 15 & 30 ml test tubes
2. Suba seals
3. Wide rubber bands
4. Vortex mixer
5. Solvent, acid and caustic dispensers and precision pipettes
6. Nitrogen evaporator
7. Nitrogen gas cylinder
8. Thermostatically controlled orbital shaking incubator plus black cloth to use as a cover
9. Thermostatically controlled heating block to fit 15 ml tubes
10. Laboratory oven
11. Pasteur pipettes

Procedures

1. Weigh 130 mg of protected lipid, untreated lipid, known standards and blanks. This should be done a day in advance, and tubes stored in the refrigerator.
2. Pipette 10 ml of strained rumen fluid into test tube.
3. One tube from each sample remains un-incubated (zero hour). Proceed to the saponification step with these.
4. Flush the remaining duplicate sample sets with nitrogen using a pasteur pipette attached to a gas cylinder via a rubber tubing.
5. Cap with suba seals and seal tightly with rubber bands to maintain anaerobic conditions.
6. Incubate samples in a shaking incubator at 39ºC for 24 hours.
7. Remove at the end of incubation, allow to cool, and carefully remove Suba seals, Proceed to the saponification step.

Saponification

1. Add 2 ml of ethanol
2. Add 2 ml of 5 N sodium hydroxide (NaOH).
3. Shake well and cover with foil.
4. Place into oven @ 80ºC for 1.5-2 h.
5. Remove from oven
6. Allow to cool.

Acidify

1. Add 5 N hydrochloric acid (HCL) approx 2 ml.
2. Invert test tube with care.
3. pH must be checked for each sample using pH paper till acid (must be pink)
4. When sample has cooled sufficiently
5. Extract fatty acids – add 4 ml of petroleum ether, shake well, pipette the supernatant into a labelled 15 ml test tube.
6. Repeat above step – pooling the extracts

7. Evaporate pooled PE extracts to dryness in a warm water bath under a stream of nitrogen.

Methylate

1. To the dried sample add 3 ml of 1% sulphuric acid in methanol (freshly made).
2. Reflux on a heating block at 50-60ºC for 1.5 h.
3. Add 3 ml of 5% NaCl (salt solution).
4. Add 2 ml of PE.
5. Cool, shake well.
6. Centrifuge at 2000 rpm for 3 minutes or allow to stand until phases clear.
7. Decant supernatant into GLC vial and cap.
8. Make sure sample vials are clearly labelled with number, date and name of operator.
9. Run on GC.

Calculation

$$\text{Protection (\%)} = \frac{\%\ C_{18:2}\ \text{after incubation}}{\%\ C_{18:2}\ \text{before incubation}} \times 100$$

Reference: Gulati S.K. (1976). Protected triacylglycerol and sterol supplements for ruminants. MSc thesis, Macquarie University, North Ryde, NSW, Australia.; Gulati, S.K., Ashes, J.R. and Scott, T.W. (1977). Assessing the degradation of fat supplements in ruminants. Anim. Feed Sci. & Tech. 64:127.

11.9 Determination of acid value

Acid value gives the measure of proportion of free fatty acids (FFA). It can be defined as mg of potassium hydroxide (KOH) required to neutralize free fatty acids present in 1 g of sample. A known quantity of (5.1000 ± 0.1000 g) sunflower acid oil was dissolved in 50 ml of neutral solvent (ether: 95 per cent alcohol: phenolphthalein = 25: 25: 1 ml and neutralized with N/10 NaOH). The contents were titrated against 0.1 N KOH in the presence of phenolphthalein as indicator. End point was the appearance of a faint pink color.

The acid value was then calculated by using the formula:

$$\text{Acid value (mg KOH/g)} = \frac{\text{Titre value x Normality of KOH x 56.1}}{\text{Weight of sample}}$$

Reference: AOAC method 969.17, 16th edition 1995.