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DETERMINING HIGHER FATTY ACID LEVELS IN PLANT MATERIALS[1]

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ABSTRACT

The higher fatty acids (HFA) are important plant constituents that are implicated in the grass tetany hazard in livestock. A method is given whereby a technician can analyze 12 forage samples daily for HFA content.

The method consists of saponifying the plant material in ethanol and KOH and extracting the subsequently acidified HFA with petroleum ether. The petroleum ether phase is evaporated and the HFA residue is dissolved in ethanol and then titrated with standardized isobutanolic KOH in the absence of O_2 by using a N_2 atmosphere. A standard plant sample, analyzed over a 37-day period, had a mean of 136 *m*mol H^+/kg ± 4.5, where a 0.1 *m*mol H^+/ℓ palmitic standard was determined with an accuracy of 99 ± 1.9%.

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INTRODUCTION

The higher fatty acids (HFA, aliphatic carboxylic acids of C_8 to C_{18}) in immature forage plants have been implicated in Mg deficiency (grass tetany, hypomagnesemia) of ruminants because of the presence of insoluble soaps of Ca and Mg in their faeces (Kemp, et al., 1966; Wilson, et al., 1969; Molloy, 1971). Although several recent studies have reported HFA values in forage (Barta, 1975; Mayland, et al., 1976; Molloy, et al., 1978), an easy-to-follow analytical method is not available in the literature. This paper describes a procedure that is easily followed and points out critical steps in the HFA determination.

This method, while basically similar to that of Immink, et al. (1965), differs from it by specifically identifying the various reagents and recommending that sample titration be done in an O_2-free environment to prevent autoxidation of the HFA. The general procedure consists of reacting tissue lipids with KOH and extracting the saponified HFA with an organic solvent. The K salt is converted to the acid form by adding HCl. The HFA are separated from the aqueous HCl, KOH, water-soluble acids, unsaponifiable lipids, and other plant constituents by petroleum ether extraction. The petroleum ether is evaporated, the HFA residue is dissolved in ethanol; and the HFA is quantitatively determined by titration with standardized KOH in the absence of oxygen.

SAMPLE PREPARATION

Sample preparation is critical because the HFA easily undergo oxidation. Molloy, et al. (1974) have thoroughly studied

sample preparation techniques for later HFA determination. They reported that the most satisfactory procedure was to freeze the intact, undried herbage at -16 C, and then freeze-dry and grind it immediately before analysis. A second, but less satisfactory, procedure was to dry the tissue at 108 C in a forced-draft oven for 45 minutes (complete drying was achieved), followed by grinding and later analysis. In the latter case, a net loss of about 1.4 mmol H^+/kg-month occurred in the grass tissue during storage. Similar oxidation losses would be expected from freeze-dried plant material. Therefore, the samples should be ground and analyzed soon after drying. Oxidation of HFA seems to be related to storage time rather than temperature. Dried samples should be ground to pass at least 40 mesh to aid in obtaining a representative sample and eluting the tissue residue from the separatory funnels.

ANALYTICAL PROCEDURE

Reagents and Materials:

Ethanol (95%) neutral

Ethanol (95%) containing 0.4 ml n-amyl alcohol/100 ml.

KOH (6.0 N).

HCl (7.8 N).

HCl (0.050 N): Standardized against primary standard. Used to determine the isobutanolic KOH titer.

Washed petroleum ether (b.p. 36 to 51 C): Wash 6 liters of petroleum ether with 1 liter of 1% aqueous KOH to remove peroxides and other oxidizing agents, elute the

aqueous KOH, and follow with two successive washings with 200-ml distilled water. After washing, the petroleum ether should be neutral against thymol blue, indicating complete removal of the KOH. If not neutral, wash again with water. The water phase can be removed by using a large separatory funnel or a small diameter water-aspiration tube of suitable length.

Isobutanolic KOH (0.040 N): Dispense with 5-ml buret equipped with Teflon[2] stopcock. Reflux 1.1 liter of isobutanol for 3 hours through a Soxhlet extractor having an extraction thimble containing 20-g NaOH. Next, dissolve 3-g KOH in 10-ml H_2O, dilute with 20-ml methanol, and add to 1 liter of the refluxed isobutanolic fraction. Distill and retain the distillate collected at 105 to 108 C, and determine its normality with 0.050 N HCl. Disposal of 100-ml of solution left after distillation at 108 C should be by evaporation.

Thymol blue: Dissolve 0.29 g in 100-ml 95% ethanol, centrifuge to remove excess indicator salts.

Cold distilled water.

Palmitic acid, or other suitable HFA standard (e.g., stearic, oleic).

Precaution:

Petroleum ether and isobutanol are extremely volatile and flammable. Do not use near open flame.

Other Materials and Laboratory Equipment:

Round-bottom flasks, 250 ml, with 24/40 $\bar{S}$ joint.

Reflux condensers, 500 mm, with 24/40 $\bar{S}$ joint, Liebig.

Electric heaters suitable for refluxing solution in 250-ml round-bottom flasks (e.g., LabCon[2] Kjeldahl digesting units).

Separatory funnels, 500 ml, with Teflon[2] stopcock and polyethylene stoppers.

Berzelius beakers, 200 ml, tall form.

Magnetic bars and stirrer.

Electric hot plate with sand bath.

N_2 gas, regulator, and O_2 scrubber.

Plastic film (e.g., Parafilm[2]).

Soxhlet extractor, condenser, a 2-liter round-bottom flask, extraction thimble, and appropriate electric heater for distillation of isobutanol.

Buret 5-10 ml Thistle tube

Standards:

Blanks and HCl standards in ethanol should be titrated daily with isobutanolic KOH to check the normality of the isobutanolic KOH. At least one HFA standard and one blank should be carried through the entire procedure daily as a further check of analytical precision. The HFA standard can be made by dissolving 0.2564-g palmitic acid in 100-ml ethanol, giving 0.01 mmol H^+/ml.

Procedure:

Weigh 0.5 to 1.0-g prepared plant material into a 250-ml round-bottom flask. Add 40 ml of ethanol and n-amyl alcohol

solution. Place the flask on electric hot plate, attach condenser unit and warm slightly. Add 10 ml of 6.0 N KOH at the top of the condenser with an automatic pipette. Wash down walls of the condenser with 4- to 5-ml ethanol and reflux for 90 min. Bumping should not occur unless the sample is near boiling before KOH addition; however, if bumping occurs, add 2 or 3 boiling stones to the flask from the top of the condenser. After 90 min., turn off heat and cool until contents have stopped boiling. Add two 20-ml aliquots of cold distilled water through the top of the condenser. Remove refluxing flask and wash bottom of condenser with 95% ethanol, collecting the rinse in the flask. Stopper and refrigerate sample for 20 to 30 min. to complete cooling.

Quantitatively transfer contents of flask to a 500-ml separatory funnel. Rinse flask with three 5-ml aliquots of neutralized ethanol and three 20-ml aliquots of cold water to ensure complete transfer. Avoid using boiling stones if possible in refluxing, otherwise care must be taken that they are not transferred to the separatory funnel with the sample. Add 10 ml of 7.8 N HCl, and swirl to mix (pH should be about 2.0). The sample will become slightly warm upon adding the HCl, but will cool as soon as petroleum ether is added.

The procedure from this point to the titration phase should be conducted in a well ventilated hood. Add 100 ml of washed petroleum ether and shake vigorously for 30 sec. Shake twice more, allowing 10 min. to elapse between each shaking. Some pressure may develop during shaking. This can be vented through

the stopcock, or may be used to assist elution of the plant material after the third shaking. About 30 sec. after the final shaking, elute the plant material that has settled in the throat of the funnel by rapidly turning the stopcock. If the plant material does not elute, shake and repeat. After removing plant material, wash down top stopper and inside of funnel with a fine spray of 95% ethanol to remove any KOH residue. Replace top and gently swirl contents. As soon as petroleum ether phase is well separated, elute and discard all but 1 cm of ethanol-KOH phase. Add 50 ml of cold water to petroleum ether and shake vigorously for 30 sec. Immediately wash top, stopper, and inside with a fine spray of 95% ethanol, then swirl gently. Allow to set for 20 min., elute all of the water phase, and discard.

Wash delivery tip of separatory funnel with 95% ethanol and discard. Elute petroleum ether phase into a 200-ml Berzelius beaker, allowing a few minutes for it to drain well. Add a 5 x 5-mm piece of Whatman[2] #42 filter paper to each beaker to aid boiling, and place in a warm sand bath. Evaporate ether phase. add 30 ml of ethanol, and dissolve residue by warming gently without boiling on the sand bath. While sample is still warm, add 5 drops of thymol blue indicator and a magnetic stirring bar, and cover immediately with plastic film, sealing the edges so they remain airtight. The HFA samples are stable at this point and may be allowed to stand several hours until additional samples are ready for titration.

Titrate with the standardized isobutanolic KOH to an intense green end point, while gently bubbling N_2 through the sample

solution. Do this by making two holes in the plastic film, one for a thin glass tube connected to the N_2 supply, the second for the buret tip which allows delivery of the isobutanolic KOH to the sample. Place beaker on a magnetic stir plate and rotate the magnetic bar while titrating.

A thistle-type buret can be used to quantitatively dispense the isobutanolic KOH, rather than the automatic-type buret, which must be connected to the titrant supply bottle. Latex[2] and Tygon[2] tubing are readily dissolved by the titrant, and even though Teflon[2] is resistant, it is very stiff and difficult to use and thus is not recommended. (Note: the recent availability of flexible Teflon tubing should be investigated.) The thistle buret can be satisfactorily filled with an automatic pipette. The buret is emptied of titrant at the end of each day and thoroughly rinsed with 95% ethanol.

RESULTS AND DISCUSSION

The 0.1-mmol standard palmitic acid was determined with an accuracy of 99 ± 1.9%. Analysis of a standard refrigerated plant sample gave a mean of 136 mmol H^+/kg with a standard deviation of 4.5, without correction for possible oxidation loss during the 37-day study period.

Standard errors encountered in experimental work include biological and analytical errors. The following is given as an example of the anticipated precision: Grass samples from a light x temperature x nitrogen greenhouse study were harvested, placed in plastic bags, and frozen at -18 C. Samples were freeze dried, weighed, and ground to pass a 40-mesh screen. After grinding,

samples were stored at -18 C until analyzed, generally within 1 week. The resulting HFA treatment means ranged from 49 to 189 *m*mol/kg with a standard mean error of 5.0 *m*mol/kg.

Previous authors (Immink, et al., 1956; Molloy, et al., 1974) have advised covering the isobutanolic KOH with mineral oil to exclude CO_2, which otherwise forms a K_2CO_3 precipitate. Our procedure allowed a gradual reduction in normality from 0.0398 to 0.0379 N KOH over the 37-day period. The rate of decline would vary with the amount of CO_2 contacting the isobutanolic KOH. We believe that the convenience of our procedure justifies the short time required to restandardize the titrant.

Using this procedure with six condensing units, six separatory funnels, and 12 round-bottom flasks, a technician can analyze 12 prepared samples per day. A few additional days are required to assemble laboratory equipment and prepare reagents. Refluxed samples and extracts of samples transferred to separatory funnels can be placed in a refrigerator overnight without loss of HFA. Extracts that have been dried and taken up in the ethanol can remain overnight if covered with plastic film. Time can often be used to advantage by weighing samples into the round-bottom flasks in the afternoon, and doing the extraction the next morning.

[2]Trade names are included for the benefit of the reader and do not imply any endorsement or preferential treatment of the product listed by The University of Arizona or U.S. Department of Agriculture.

REFERENCES

1. Barta, A. L. Crop Sci. 15:169-171. (1975).

2. Immink, H. J., J. H. Guerink, and W. B. Deijs. Jaarbaek, Int. Biol. Scheik., p 103-107. (1956).

3. Kemp, A., W. B. Feijs, and W. B. Kluvers. Netherlands J. Agric. Sci. 14:290-295. (1966).

4. Mayland, H. F., L. F. Molloy, and T. W. Collie. Agron. J. 68:979-982. (1976).

5. Molloy, L. F. N. Z. Soil Bureau Scient. Rept. No. 5 (1971).

6. Molloy, L. F., Roger Ball, T. W. Collie, and D. J. Ross. N. Z. J. Agric. Res. 21:57-74. (1978).

7. Molloy, L. F., D. J. Giltrap, T. W. Collie, and A. J. Metson. J. Sci. Food Agric. 25:595-606. (1974).

8. Wilson, G. F., C. S. W. Reid, L. F. Molloy, A. J. Metson, and G. W. Butler. N. Z. J. Agric. Res. 12:467-488. (1969).