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A SIMPLE AND QUICK ON-FARM TOOL KIT TO DETERMINE NITRATE LEVELS IN FORAGES AT FIELD CONDITIONS

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Nitrate poisoning, mainly due to consumption of excessive amount of nitrate from forages, hay, silage, weeds and drinking of water or waste water, is one of the problems faced by dairy industry in Sri Lanka. Though few laboratory methods are already available for the quantification of nitrate levels in forage, a new on-farm technique or method is required to practice at field condition. An experiment was conducted to find out the suitability of introducing a new method to determine the forage nitrate levels at field conditions. The new method was developed with series of experiments and validated with the standard laboratory Salicylic Acid method. A field experiment was conducted in a Randomized Complete Block Design (RCBD) in two factor factorial arrangement with three replicates to evaluate the effect of plant parts (stem and leaves) and growth stages (15 & 30 and 45 days) on nitrate accumulation using the developed method. Data were statistically analyzed using paired t-test and two way Analysis of Variance (ANOVA) in SAS. Results revealed that the developed method was significantly different ($P<0.05$) to standard laboratory Salicylic Acid method. However, there was a linear relationship between two methods. Accordingly, a value of 398.1ppm nitrate concentration needs to be adjusted with the new method. In addition, it showed an interaction between two factors in nitrate accumulation ($P<0.05$). The highest nitrate contents were observed in stems compared to the leaves and declined with maturity. Thus, it can be concluded that the developed method can be used at field conditions to measure the nitrate level with an adjustment and nitrate accumulation in forages depends on plant parts and growth stage.

Keywords: Nitrate poisoning, Plant factors, Salicylic acid method

Pasture and fodder play a vital role in the livestock nutrition, especially in ruminants because it is the cheapest source of animal feed. In Sri Lanka, approximately 12,000 km^2 out of the total land extend are under grasslands (Premaratne *et al.*, 2003), which consist with natural and improved grasses. There are about 20,000 ha of improved grasses which are mainly found in the government farms (NARESA, 1991). However, small and medium scale farmers fulfill their pasture requirement from natural lands such as common lands on road sides, railway banks, fallowed paddy fields, tank beds and other vacant lands. Guinea grass, *Brachiaria* spp, fodder sorghum, maize, Napier and its hybrids are the main pasture and fodder species used by the farmers. CO-3 is one of the Napier hybrid, introduced to Sri Lanka by Livestock Breeding Project (LBP) in 1999 (Premaratne and Premalal, 2006). Many farmers have already accepted to cultivate CO-3 under the government promotion programs due to its characteristic features of high tillering, high yield potential, high dry matter (15-16%) and crude protein (9.8 - 12.8%) content, quick regeneration capacity, high leaf to stem ratio, high palatability, resistance to pest and diseases and low in adverse factors (Premaratne and Premalal, 2006). But, some problems have arisen with cultivation of fodder varieties in local farms mainly due to nitrate poisoning which is one of the problems faced by dairy industry in Sri Lanka.

Nitrate poisoning is one of the major problems which take place in the dairy industry of Sri Lanka mainly due to high accumulation of nitrates in fodder and pasture. Nitrate poisoning in cattle is caused by the consumption of excessive amount of nitrates or nitrites from grazing crops, hay, silage, weeds, drinking water and waste water (Vough *et al*., 2006). The excessive amounts nitrate accumulation occur in forages when have been grown under conditions of excessive fertilization and/or stress. However, plant species and adverse environmental conditions before harvest affect the concentration of nitrates even more than available nitrogen in the soil. Direct ingestion of fertilizers that contain nitrates can be toxic to livestock. Any stress condition which causes an abrupt decrease in plant growth may contribute to plant nitrate accumulation, even with a normal nitrogen supply (Crawford *et al*., 1961). Most forages contain small amount of nitrate which is not particularly toxic to cattle. When feeds contain more nitrates which is eaten by ruminants, the nitrate is normally broken down to ammonia and converted by bacteria into microbial protein. Nitrite, one of the intermediate products involved in this process, is the cause of "nitrate poisoning." Some of the nitrite is absorbed into the animal's bloodstream where the nitrite is capable of changing the normal oxygen transporting substance, hemoglobin, into met-hemoglobin, a substance that cannot carry oxygen from the lungs to the tissues (Vough *et al*., 2006). Many species are susceptible to nitrates and nitrite poisoning, but cattle are affected most frequently. Ruminants are especially vulnerable because the ruminal flora reduces nitrate to ammonia, with nitrite (~10 times more toxic than nitrate) as an intermediate product. Young pigs also have GI micro flora capable of reducing nitrate to nitrite, but mature monogastric animals are more resistant to nitrate toxicity because this pathway is age-limited (Rahman *et al.,* 2011).

Nitrate is the primary nutrient form of nitrogen in most soils and is a normal constituent of plants. Normally nitrate is assimilated so rapidly following absorption from soil that its concentration in plant tissues is low. Occasionally, excessive levels in plants occur. At present, the most notorious accumulators of nitrate are the sorghums. Other annuals that less frequently accumulate nitrate are small grains (wheat, oats, rye and barley) and millet. Some perennial grasses CO-3, Rye, Guinea also contain dangerous levels. Accumulation is usually triggered by some environmental stress where plant growth is restricted but absorption of nitrate from soil continues. Lack of moisture, together with excessive soil nitrogen for existing growing conditions, is a frequent cause of toxic levels of nitrate in sorghums. Other stress factors that favor buildup are reduced sunlight from cloudiness or shading, frost, certain herbicides including 2,4-D, acid soils, low growing temperatures, and deficiencies of essential nutrients like phosphorus, sulfur and molybdenum. When more soil nitrogen is present than needed for maximum growth, some plants tend to accumulate nitrate even without environmental stress. This response is particularly true with hardy soil feeders like sorghum, noted for "luxury consumption" of certain nutrients. When accumulation occurs, the concentration of nitrate is greater in stems than leaves. Seeds seldom contain significant amounts. Rate of uptake diminishes with maturity; mature plants usually contain less nitrate than immature ones. Differences in potential for accumulation exist among species and varieties (Undersander *et al*., 2006).

Nitrate poisoning has become one of the major issues in the Ambewela dairy farm, Sri Lanka. As per the farm records, it has been estimated that more than 40 cows have died every year due to nitrate poisoning under local condition. There are number of laboratory methods available to identify the nitrate level of the forages in the field. These laboratory methods are expensive, laborious and time consuming process. However, there is no any nitrate identification tool kit at the field level. Therefore, the present experiment was carried out to introduce a simple and quick on-farm tool kit for determination of nitrate levels of forages at the field level.

MATERIALS AND METHODS

Location

Experiment was conducted at two locations including dairy farm land, Ambewela and pasture land, VRI. At least 20 pasture samples from the pasture land was randomly selected between the weights of 0.5-1kg. A total 300 samples was selected to represent the whole farm land. It was assumed that selected samples showed a fair representation of the pasture land and dairy farm land.

Data Collection and Sampling Procedure

Primary data (Nitrate ppm level) collection

The following methods were used to measure nitrate levels at laboratory condition and compared the values.

- Standard Laboratory Salicylic Acid method was done using spectrophotometer.
- Quick Nitrate Test kit (QNT) method was used under the laboratory condition using spectrophotometer.

Sample collection

Already established fields of hybrid Napier var. CO-3 and fields of Rye grass (Var. Aston) were selected from each farm. Recommended plot area for each farm was demarcated (Length × width = 5 m × 5 m =25 m^2). All plots were harvested at 15, 30 and 45 days after the establishment of forage.

Experimental design

The experiment was conducted as two factor factorial arrangement with three replicates to evaluate the effect of plant parts (stem, leaf) and growth stages (15,30 and 45 days) on nitrate accumulation.

Sample preparation

The harvested forage samples were separated as stem and leaves and cut into small pieces. Then, 20 g of each stem and leaves samples was grounded using field grinder with 200 ml of distilled water.

Laboratory analyse

Each forage sample was analysed for nitrate levels using Standard Laboratory Salicylic Acid (Catalado *et al*., 1975) and Quick Nitrate test kit (QNT) methods. The QNT is the new method which was developed with series of experiments and validated with the standard laboratory Salicylic Acid method. Further, QNT colour chart and working procedure were developed with series of experiments to use at the field level.

Developed method

Quick Nitrate Test kit (QNT) method

Sample

⇩

Separate into leaves and stem

⇩

Cut into small pieces

⇩

Get 1g from sample

⇩

Put into centrifuge tube and add 10ml of

⇩

distilled water

⇩

Shake well approximately 30second

⇩

Get 1ml from centrifuge tube and pour in to

⇩

test tube

⇩

Add 1ml of nitrate regent #1

⇩

Add 200mg of nitrate regent #2

⇩

Shake well and keep it 2 minutes

⇩

Add 10ml of distilled water

⇩

Centrifuge it 600rpm – 5minutes

⇩

Get absorption of nitrate using Laboratory

⇩

Spectrophotometer

⇩

Convert absorption into nitrate ppm levels

Extraction of forage juice for nitrate analyse

Plant juice + nitrate regent #1 + nitrate regent #2

Centrifuged solution

Take absorption of nitrate at 540nm

The developed QNT kit is composed with,

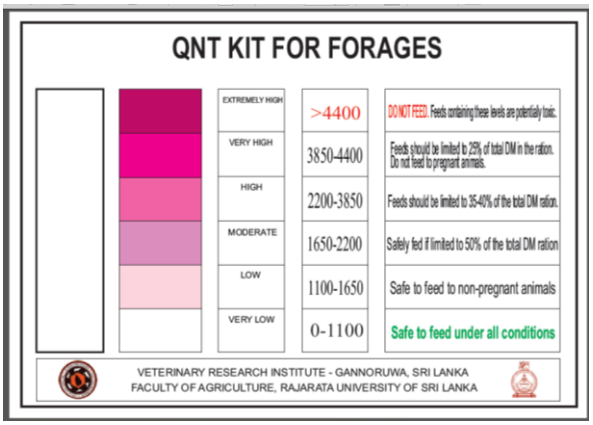
- Distilled water bottle
- Nitrate regent #1
- Nitrate regent #2
- Four Centrifuge tubes
- Four test tubes
- Porcelain plate
- Small 200 mg spoon
- Four droppers
- Scissor
- QNT colour chart
- QNT working procedure

QNT kit

The developed working procedure of QNT kit

- Take the forage sample with stem and leaves
- Separate the forage sample into stem and leaves
- Cut the forage stem or leaves into small pieces separately using scissor or knife
- Collect small parts into porcelain plate
- Put approximately 1g of collected pieces into the 15 ml centrifuge tube and mark the upper most point of the centrifuge tube using red color band
- Then, add distilled water to the centrifuge tube up to 10 ml and mark the point using black color band
- Tight the tube lid and shake well approximately 30 seconds
- Then, open the lid and take 1ml forage juice from tube and pour into the test tube
- Add 10 drops or 1ml from the nitrate regent # 1_ Liquid form which is used for color enhancer
- Then, add 200 mg from the nitrate regent # 2 _ Powder form which is used for color development
- Close the test tube mouth using the finger and shake well
- Keep tube 3 minutes
- Compare developed colour with the developed QNT colour chart and read the value and recommendations.

Developed QNT Colour Chart



Data analysis

Paired t-test procedure of SAS was used to compare the QNT kit and standard laboratory salicylic acid methods. Regression line was built up using regression procedure of SAS to show the linear relationship between above two methods. Randomized Complete Block Design (RCBD) was used in two factor factorial arrangement with three replicates to evaluate the effect of plant parts (stem, leaf) and growth stages (15,30 and 45 days) on nitrate accumulation. Significance was declared at p=0.05.

RESULTS AND DISCUSSION

Nitrate accumulation in plant parts

There was a significant association (P<0.05) between two factors such as plant part and growth stage on nitrate accumulation.

Table 1 shows the means of nitrate concentration (% wet basis) in forage samples between two farms. Nitrate content in plant parts significantly differed (p<0.05) between stem and leaves. Stems showed a higher nitrate content compared to leaves. Nitrate accumulation in fodder may vary depending upon the genetic variability on nitrate uptake by the plants. The amount of

Table 1: Means of nitrate concentration (ppm, %wet basis) of plant parts between two farms at different maturity stages.

Plant parts	Maturity (days)	VRI Pasture Land	Ambewela Farm
Stem	15	1057.20±220.15[a]	1626.14±311.34[a]
	30	3946.07±233.51[a]	5846.38±330.23[b]
	45	284.71±183.18[a]	388.79±259.05[a]
Leaf	15	488.25±311.34[a]	715.56±246.35[b]
	30	2045.75±330.23[a]	2888.87±320.92[b]
	45	180.63±50.36[a]	307.62±174.19[b]

Data are presented as mean ± SD

Means within the same row with same superscript are not significantly different (p>0.05)

nitrate accumulated within the plant depends upon the rate of nitrogen uptake by the plant from the soil and rate of its reduction in the plant. Plant parts are highly affected on the nitrate accumulation in forages. The difference of nitrate levels in stem and leaves occurred similarly due to leaves act as primary site of converting nitrate into protein and difference in ability of roots to take up nitrogen from soil (Glunk *et al.*, 2015). Rasby *et al.,* (2007) and Sidhu *et al.,* (2011) reported that, nitrate content is highest in stem tissue, followed by leaf tissue. Findings of the present study have confirmed that nitrate content differs with the plant parts which contain more nitrates close to the ground and along the length of plant, nitrate content decreases.

Nitrate accumulation on growth stages

The changing pattern between maturity stages at 15, 30 and 45 days versus plant nitrate level is give in the table 1. There was a significant difference between plant maturity stage and nitrate levels (P<0.05).With the maturity, the forage nitrate levels was decreasing. In the present study, highest nitrate level showed at the age of 30 days (P<0.05) than the 15 and 45 days. Among 15 and 45 days of age, at 15 days, plants showed the highest nitrate concentration in plant body. The difference may be due to genetically pattern and lower management practice were taken with the age and climatic parameters also affect to the forage nitrate levels. Lower nitrate levels in mature plants may be due to decreased uptake or increased enzyme activity to convert the nitrate into intermediate compounds ready for evaporation or used by the plant. The results confirmed the previous findings reported by Rasby *et al.,* (2007) and Sindhu *et al.,* (2007) where they observed that nitrate levels reduced with the maturity of plants. Moreover, data suggested that nitrate content decreases with plant maturity. Thus, delaying the harvesting stage up to standard may reduce the toxic effect of nitrate.

Comparison between standard salicylic acid method versus developed QNT Kit

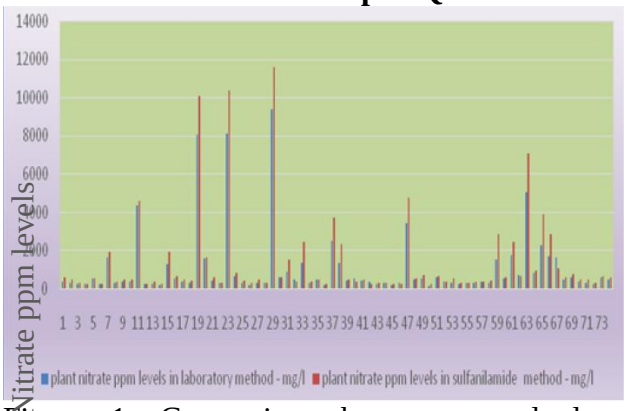


Figure 1: Comparison between standard salicylic acid methods versus developed QNT Kit (sulphanilamide method)

Standard salicylic acid method and quick nitrate test kit were used to measure the nitrate level in forages (wet basis). But, Standard salicylic acid methods can only be used under laboratory conditions. Quick nitrate test can be used both at field condition as well as under laboratory conditions with few adjustments. Figure 1 shows the nitrate ppm level obtained in laboratory using spectrophotometer. In addition, data analysis showed a significant deference between two methods (P<0.05).

The least significant difference between two methods was estimated as 398.1 ppm. A regression line between these two methods was built up because of the significant difference between two.

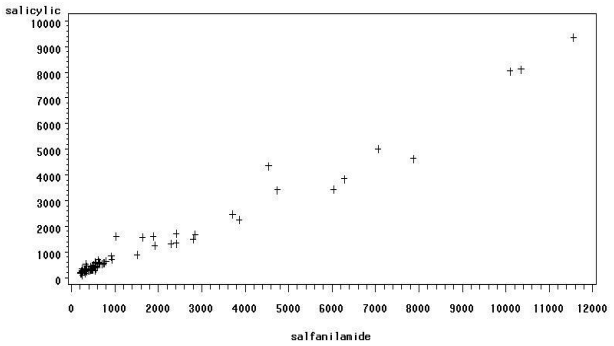


Figure 2. Regression line between standard salicylic acid methods versus QNT Kit

Figure 2 shows the linear relationship between these two methods using regression line. According to the regression line, the following equation was derived for these two methods.

$$Y=0.729X+18.068$$

Y= Old method spectrophotometer absorption at 410nm
X = New method spectrophotometer absorption at 540nm

These two methods illustrated the difference in nitrate concentration under similar laboratory conditions due to the powder form of the chemical (nitrate regent#2) that was used in Quick nitrate test kit. When Quick nitrate test kit was used in laboratory conditions using spectrophotometer, it was read the absorption with higher values due to this powder form (nitrate regent#2). This was the main reason for the difference between two methods. However, filtrating could be solved this problem easily. But at field level, it should not become a huge problem since we are not using spectrophotometer. Further, adjusted colour chart has been developed for the use at field conditions.

CONCLUSIONS

The nitrate content in forages varies depending upon the forage maturity stage and parts of the plant. Forage stem has the highest nitrate content with compared to the leaves. Nitrate content declines with the plant maturity.It is also desirable to use correct harvesting practice to reduce nitrate content in forage for the acceptable level, because nitrate content in forage declines with maturity. Forages after harvesting should be kept at least 2 hrs under sunlight to lower the nitrate content. The developed method (QNT kit) is easiest and quick on-farm technique that could be recommended for the determination of nitrate levels at the field condition within 2 minutes.

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