Correlation between Pummelo Leaf Nitrogen Concentrations Determined by Combustion Method and Kjeldahl Method and their Relationship with SPAD Values from Portable Chlorophyll Meter

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ABSTRACT

Discrepancies in reports of leaf tissue nitrogen (N) concentrations are in part due to different analytical methods among laboratories. Two standard methods of N determination, the combustion method and the Kjeldahl method, were compared for pummelo [Citrus maxima (Burm. f.) Merr.] leaf N analysis. Pummelo plants were fertilized with different N rates and leaves with different ages were sampled to obtain various leaf N levels. Leaf greenness (SPAD value) of each sample was measured by a portable chlorophyll meter prior to nitrogen analysis. The results showed that N concentrations of pummelo leaf samples were in the range 1.23-2.54% using the combustion method and 1.00-2.08% for the Kjeldahl method. The combustion method yielded higher N concentration values than the Kjeldahl method in every sample. A linear relationship between the combustion and Kjeldahl N values was highly significant (Kjeldahl N (%) = 0.7829 \times \text{Combustion N} (%) + 0.1150; R^2 = 0.86 and \( p \)-value < 0.0001). The addition of samples of pummelo fruit parts that had wider ranges of N concentrations (0.74-4.08% for the combustion method and 0.57-3.51 % for the Kjeldahl method) than leaf tissue into the analysis increased the coefficient of determination (Kjeldahl N (%) = 0.8676 \times \text{Combustion N} (%) – 0.0384; R^2 = 0.96 and \( p \)-value < 0.0001). Therefore, comparisons and interpretation of pummelo leaf N analysis results from different laboratories should mention the analytical method used and provide a linear equation for adjusting the N values. In addition, SPAD values had a positive correlation with leaf N concentrations determined by both methods and could be explained by quadratic polynomial equations (Combustion N (%) = 0.0003 \times \text{SPAD}^2 – 0.0313 \times \text{SPAD} + 2.5024; R^2 = 0.21 and \( p \)-value < 0.0001; Kjeldahl N (%) = 0.0004 \times \text{SPAD}^2 – 0.0372 \times \text{SPAD} + 2.4086; R^2 = 0.16 and \( p \)-value < 0.0001). It is suggested that the SPAD value determined by a portable chlorophyll meter can be used to obtain a quick estimation of pummelo leaf N status.

Keywords: Dumas-N, Kjeldahl-N, leaf greenness

INTRODUCTION

Awareness of leaf analysis as a tool for efficient nutrient management is increasing among fruit growers in Thailand. Leaf nutrient analysis results are compared to published leaf nutrient concentration standards to determine whether fruit trees have the proper nutritional status (Mills and Jones, 1996) and the optimum amount of fertilizer to be applied to achieve potential yield, fruit quality and sustainable tree health. The Kjeldahl method is commonly used for nitrogen (N) analysis of leaf
tissue and most of the standard leaf N concentrations published for several fruit crops were established by this method. The Kjeldahl method consists of a digestion step of dried leaf tissue samples using concentrated acid under high temperature conditions, followed by the quantification of the nitrogen released from the samples as ammonia by titration, an ion-specific electrode or colorimetry (Jones, 1991). By nature, the Kjeldahl method only converts protein-N and some nitrate-N to ammonia. Therefore, the Kjeldahl method can underestimate the total N concentration of leaf samples with high nitrate accumulation (Simonne et al., 1998). The combustion or Dumas method is another method for N analysis that has been used increasingly for routine diagnosis of N in plants, food and feedstock, due to the advances in N-analyzers, which require less determination time for each sample and the diminished cost of safely disposing of hazardous waste chemicals, as compared to the traditional Kjeldahl method. In the combustion method, dried leaf samples are burned completely in the induction furnace of the N-analyzer, where organic-N and inorganic-N are transformed into NO\textsubscript{x} gases, which are then reduced to N\textsubscript{2} gas. Amounts of N\textsubscript{2} are then measured automatically by thermal conductivity. Because of its nature, the combustion method determines truly the total N in the leaf sample. Thus, substantial differences in reported leaf N concentrations can be observed among laboratories using different analytical methods. Relationships between Kjeldahl-N and combustion-N in plant tissues, seeds, fibers, feedstock, manure and sewage sludge have been studied and the results showed that the combustion method generally yielded higher N concentrations than the Kjeldahl method, but the differences varied among types of samples and their nitrate concentrations (Simonne et al., 1994, 1995, 1998; Watson and Galliher, 2001).

The leaf chlorophyll concentration is often well correlated with leaf N status and photosynthetic rates (Evans, 1983). A portable chlorophyll meter (SPAD-502, Minolta Co. Ltd., Japan) has been considered satisfactory for predicting leaf chlorophyll and N levels of field crops, such as rice (Takebe and Yoneyama, 1989), maize (Wood et al., 1992) and tall fescue forage grass (Kantety et al., 1996). Chlorophyll meter readings (SPAD values) are instantaneous and do not involve destructive sampling. These advantages provide a rapid, quantitative and inexpensive in-field estimation of leaf N status for proper N management. However, the correlation between SPAD values and leaf N status is relatively low in woody plant species, such as red maple (Sibley et al., 1996), benjamin fig and cottonwood (Loh et al., 2002) and coffee (Netto et al., 2005), while the strong relationship between SPAD values and chlorophyll concentrations was maintained. These weak relationships can be due to the greater complications of the N source-sink relationships of perennial plants (Wood et al., 1993), leaf thickness (Peng et al., 1992) and the stage of development (Loh et al., 2002).

Pummelo is one of the economic fruit crops in Thailand and its superior fruit quality is well recognized worldwide. Unfortunately, a standard leaf nutrient concentration for Thai pummelo has not been established. In a previous study, leaf N concentration of healthy ‘Khao Nam Phueng’ pummelo trees varied with leaf ages and the values determined by the combustion method were in the range 2.96-3.22 % and 3.08-3.21% for January flushes and June flushes, respectively (Jaroonchon, 2009). These leaf N values were relatively higher than those reported for pummelo in previous studies using the micrco-Kjeldahl method for N analysis (Suriyapan, 1997; Maneepong, 2008). For proper interpretation of leaf N analysis and further development of a standard leaf N concentration for Thai pummelo using collective data from different laboratories, the correlation between the results from the Kjeldahl and combustion methods needs to be
studied. In addition, the relationship between chlorophyll meter readings and the leaf N concentration of pummelo has not been reported.

The objective of this experiment was to construct a correlation between leaf N concentrations determined by the Kjeldahl and combustion methods and the feasibility of using a portable chlorophyll meter (SPAD-502) to estimate the leaf N levels of pummelo.

MATERIALS AND METHODS

Three-year-old ‘Khao Nam Phueng’ pummelo plants were grown in a 16-inch diameter plastic pot consisting of rice husk, rice husk charcoal, loamy soil and farm yard manure (1:1:1:0.5 parts by volume) as the growing medium. Plants were separated into three groups; low nitrogen, moderate nitrogen and high nitrogen. Each group contained six uniform plants. Chemical fertilizer was omitted for plants in the low nitrogen group, while 300 ppm of 20N:20P₂O₅:20K₂O fertilizer solution, 1 L per plant or 300 ppm of 20N:20P₂O₅:20K₂O fertilizer solution, 1 L per plant in combination with foliar spray of 1% urea were applied weekly to samples in the moderate nitrogen group and the high nitrogen group, respectively. New shoots with 7-12 leaves emerging after fertilizer application were tagged, and measurements were taken when these new leaves were fully expanded. Leaf samples were taken three times from existing leaves (the first sampling time) and fully expanded leaves of various ages (1-3 months old) from new shoots (9-10 new shoots from each group), in order to get a wide range of leaf N concentration and leaf greenness values. Leaf chlorophyll concentration of intact leaves was estimated by a portable chlorophyll meter (SPAD-502, Minolta Co. Ltd., Japan) and expressed as a degree of leaf greenness or SPAD value. Then, leaf samples were harvested, rinsed with 0.1 N HCl and distilled water, respectively and oven-dried at 70°C for 48-72 h until their dry weight was constant. Dry samples were ground to pass a 40 mesh screen for further N analysis by the Kjeldahl and combustion methods.

Nitrogen determination by the Kjeldahl method

A 0.25 g ground sample was digested with 5 mL of concentrated H₂SO₄ at 380°C using Na₂SO₄ and Se as catalysts, until the sample solution was clear. The volume of each digested sample was adjusted to 50 mL by adding distilled water and 10 mL of the sample was distilled according to a standard Kjeldahl protocol, after adding 1 mL of 40% NaOH solution. A distilled sample was titrated with a standard solution of 0.02 N H₂SO₄ until the end point and the N concentration of the sample was calculated using Equation 1:

$$\text{Total N concentration in a sample (\%) = } \frac{(V_s-V_b) \times N \times 0.014 \times V_d \times 100}{W \times V_a}$$  \hspace{1cm} (1)

where

- \(V_s\) = amount of a standard H₂SO₄ (mL) used for titration to reach end point,
- \(V_b\) = amount of a standard H₂SO₄ (mL) used for titration of the blank,
- \(N\) = H₂SO₄ concentration (0.02 N),
- \(V_d\) = amount of digested sample solution (mL),
- \(W\) = sample weight (g) and
- \(V_a\) = amount of a sample solution for the analysis (10 mL)

Nitrogen determination by the combustion method

A 0.25 g ground sample was encapsulated in N-free tin foil (LECO Corporation, USA) and combusted in the furnace of a protein/nitrogen determinator (LECO FP-528, LECO Corporation, U.S.A) at 850°C. Combustion products in the gas phase, including CO₂, H₂O, NOₓ and N₂, were collected and passed to hot copper wire by a helium carrier gas to remove excess oxygen and reduce any NOₓ to N₂ gas. CO₂
and H₂O were later removed by sodium hydroxide on a silicate carrier (Lecosorb®, LECO Corporation, USA) and magnesium perchlorate (Anhydrone®, LECO Corporation, USA) and the remaining combustion product, nitrogen, was measured by a thermal conductivity cell. The total nitrogen concentration of the sample was expressed as a percentage.

Data were subjected to regression analysis (SAS Institute, 1989) to construct a relationship between the nitrogen concentration values determined by both methods. As leaf N concentrations varied generally within a narrow range, an additional 31 pummelo fruit samples (outer peel (flavedo), inner peel (albedo), carpel membrane and juice vesicles) from another study with a wider range of tissue N concentrations were included to the further regression analysis. The relationship between leaf greenness or SPAD values and leaf N concentration was also determined by regression analysis.

RESULTS AND DISCUSSION

The leaf N concentration of ‘Khao Nam Phueng’ pummelo in this study varied from 1.00 to 2.08%, as determined by the Kjeldahl method and from 1.23 to 2.54%, as determined by the combustion method. Samples of young fully expanded leaves (< 2 months old) fell into the low N concentration range. N deficiency symptoms were not evident in this study. Samples from the medium and high N groups in this study (2-3 month-old leaves) had slightly lower leaf N concentration than those previously reported from pummelo trees bearing under orchard conditions (Jaroonchon et al., 2005). The differences were in part due to the younger leaf age and juvenility of the tested plants in this study. The N concentration values from both methods had a strong linear relationship, with a high coefficient of determination value (R² = 0.86 and p-value < 0.0001) (Figure 1) and could be explained by a linear equation (Equation 2):

\[ \text{Kjeldahl N} \% = 0.7829 \times \text{Combustion N} \% + 0.1150 \quad (2) \]

All data points were below the 1:1 line, indicating that the combustion method produced higher N concentration values than the Kjeldahl method for every leaf sample. This finding agreed with a previous report on different vegetable leaves, where the combustion method yielded

![Figure 1](image-url)

**Figure 1** Relationship between N concentrations (leaf tissue) determined by the Kjeldahl and combustion methods.
higher N concentration values over the Kjeldahl method by 25% (Simonne et al., 1998). In the combustion method, samples were completely burned at very high temperature and the majority of N gas from the samples could be detected, while in the Kjeldahl method, nitrate-N (NO₃⁻) in samples may not have been completely converted to NH₃ gas during the digestion step, resulting in a lower yield of N in the analysis (Lee et al., 1996; Simonne et al., 1998).

When the parts of the pummelo fruit tissue that had a wider range of N concentrations than the leaf tissue were included into the analysis, the N concentrations were in the range 0.57-3.51%, as determined by the Kjeldahl method and in the range 0.74-4.08%, as determined by the combustion method. The combination of leaf and fruit tissues provided a stronger linear relationship (R² = 0.96 and p-value < 0.0001) between the two methods than by using only leaf samples (Figure 2). The combustion method yielded higher N concentration values than the Kjeldahl method in all leaf and fruit part samples across the range of N concentrations observed. As the N concentration in the samples increased, data points deviated away from the 1:1 line (Figures 1 and 2) suggesting that a greater discrepancy in analytical results would be of concern for samples with high tissue N concentration. The relationship between N concentrations in leaf and fruit tissues determined by both methods could be expressed as a linear equation (Equation 3):

\[ \text{Kjeldahl N (\%)} = 0.8676 \times \text{Combustion N (\%)} - 0.0384 \]  

(3)

The SPAD values of leaf samples from low, medium and high N groups were in the range 45-83. Samples with high SPAD values were mature leaves, which were relatively thick with dark green color. The relationship between SPAD values and pummelo leaf N concentrations was significant (Figures 3 and 4), although the coefficient of determination was not high (R² = 0.21; p-value < 0.0001 for the Kjeldahl method and R² = 0.16, p-value < 0.0001 for the combustion method, respectively). Similar significant relationships with relatively low R² values had been reported in hardwood species, including sweet gum (R² = 0.37), sycamore (R² = 0.57), swamp cottonwood (R² = 0.32) and green ash (R² = 0.72) (Chang and Robinson, 2003). At a certain developmental stage, N may accumulate in leaf tissue as nitrate, which does not contribute to

Figure 2  Relationship between N concentrations (leaf and fruit tissues) determined by the Kjeldahl and combustion methods.
chlorophyll and the SPAD values may underestimate the existing amount of leaf total N, resulting in a low correlation (Loh et al., 2002). The result of the current study suggested that non-destructive estimation of pummelo leaf N status can be rapidly determined using a portable chlorophyll meter. The relationship between SPAD values and leaf N concentrations could be explained by quadratic polynomial equations (Equations 4 and 5):

\[
\text{Kjeldahl N (\%)} = 0.0004 \times \text{SPAD}^2 - 0.0372 \times \text{SPAD} + 2.4086 \\
\text{Combustion N (\%)} = 0.0003 \times \text{SPAD}^2 - 0.0313 \times \text{SPAD} + 2.5024
\]

**CONCLUSION**

A strong linear relationship between leaf N analysis values from the Kjeldahl method and combustion methods was observed in ‘Khao Nam

![Figure 3](image3.png) **Figure 3** Relationship between N concentrations (leaf tissues) determined by the Kjeldahl method and leaf SPAD values.

![Figure 4](image4.png) **Figure 4** Relationship between N concentrations (leaf tissues) determined by the combustion method and leaf SPAD values.
Phueng’ pummelo and the linear equation was proposed as: Kjeldahl N (%) = 0.7829 \times \text{Combustion N} (%) + 0.1150 (R^2 = 0.86 \text{ and } p\text{-value < 0.0001}). The combustion method generally yielded slightly higher N concentrations than the Kjeldahl method throughout the range of N levels in leaf samples. A rapid and nondestructive field monitoring of N levels in pummelo leaves is feasible by using a portable chlorophyll meter (SPAD-502, Minolta Co. Ltd., Japan). The leaf N concentration of ‘Khao Nam Phueng’ pummelo can be predicted from SPAD values using the equations: Kjeldahl N (%) = 0.0004 \times \text{SPAD}^2 - 0.0372 \times \text{SPAD} + 2.4086 (R^2 = 0.21; \text{p-value < 0.0001}) and Combustion N (%) = 0.0003 \times \text{SPAD}^2 - 0.0313 \times \text{SPAD} + 2.5024 (R^2 = 0.16, \text{p-value < 0.0001}), respectively.

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