Minimization of error in leaf analysis sampling sand analysis[1]

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Research has shown that, when carried out correctly, leaf analysis provides a good indication of palm nutritional status. All plantations therefore place great emphasis on the results of leaf and rachis sampling and analysis as the basis for determining fertilizer recommendations to correct nutrient deficiencies. Because fertilizers represent such a large proportion of operational costs and have a major impact on yield, it is important that leaf sampling and analysis is based on correctly implemented procedures. Yet many plantations take insufficient care to verify that laboratory analysis is accurate and precise.

Leaf sampling and analysis process involves six main steps, each of which is prone to error. Errors in the leaf sampling and analysis process (E_{lsa}), are cumulative and may arise from a combination of errors in LSU block set up (E_{block}), LSU palm identification (E_{palm}), selection of leaf 17 (E_{leaf}), sample preparation at the plantation (E_{prep}), laboratory error (E_{anal}), and data processing (E_{data}).

$$E_{lsa} = E_{block} + E_{palm} + E_{leaf} + E_{prep} + E_{anal} + E_{data}$$

It is essential to minimize the errors at each stage of the process in order to achieve representative, accurate and precise results that can then be used to determine fertilizer requirements. Without proper procedures and cross checks, error may be as great as 10–20%, leading to 'false positives' (i.e., deficiencies reported where none exist) and 'false negatives' (deficiencies exist but are not reported). Both will affect the amount of fertilizer recommended, yields and profits.

In this paper we review common sources of error in the six stages in the process of leaf sampling and analysis and how they can be controlled.

1. Use of leaf sampling unit (LSU) blocks

In the past, leaf sampling units (LSUs) were defined as 'a set of blocks so similar in terms of soil, planting material, palm age and field conditions that it can be considered a single unit for the purpose of fertilizer recommendations'. Leaf sampling was then carried out in a single block that was considered representative for the respective LSU.

Nowadays it makes more sense to assess palm nutrient status and fertilizer requirements in each block because:

- The cost of leaf sampling and analysis is small by comparison with the cost of fertilizers.
- Block-by-block assessment provides the means for greater discrimination on fertilizer rates.

2. Identification of LSU palms

The results of leaf analysis may be distorted if there are errors in palm sampling procedures:

If different palms are sampled each year, the differences in leaf nutrient status between years and blocks may be due to palm selection rather than block nutritional status. For this reason, leaf sampling should be carried out on a grid of permanently selected and marked LSU palms (Table 1).

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- LSU palms are marked in triangular pattern, every n^{th} palm in every n^{th} row (Figure 1). The triangular pattern provides more even distribution of LSU palms and makes sampling easier and more efficient.

Table 1. Leaf sampling unit palm selection by planting system.

Planting system	Sampling grid
Flat lined planting where most blocks are rectangular shaped	Triangular grid with every 10^{th} palm in every tenth row.
Contour planting	Every 10^{th} palm along every 10^{th} contour terrace.
Low-lying areas with drains every four palm rows	Every 8^{th} palm in every 8^{th} row.

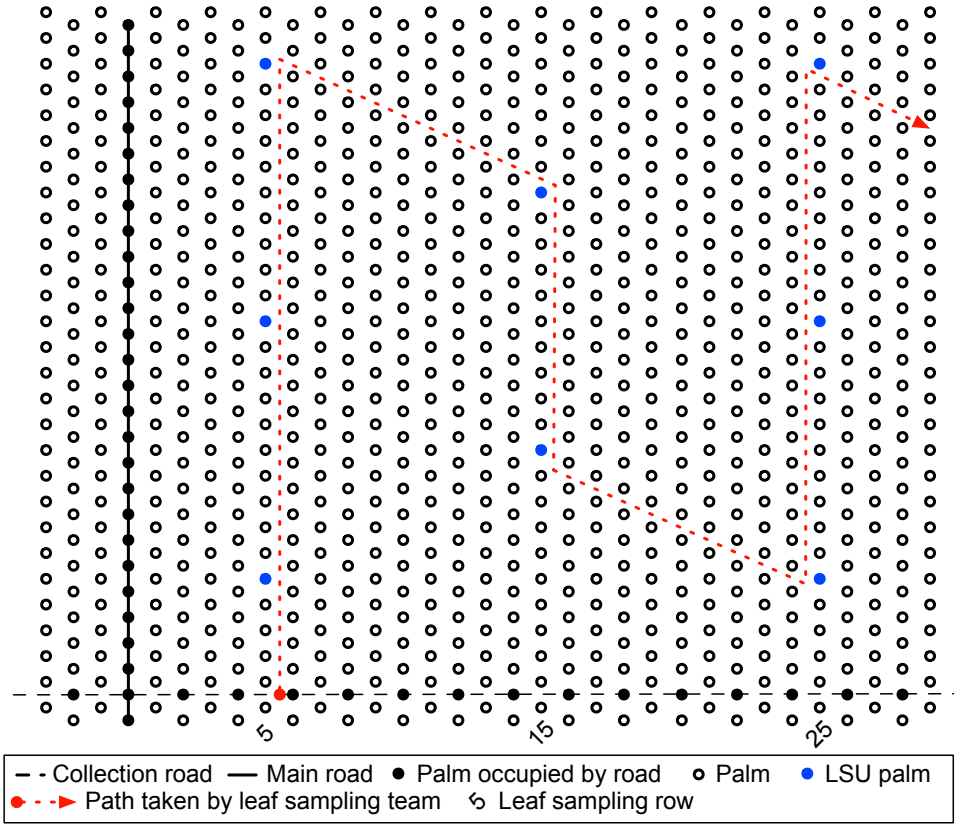

Figure 1. Location of LSU palms in a rectangular-shaped block planted on flat land.

- LSU palms must be at least four palms distant from the roadside (to avoid 'roadside bias' and contamination from dust).
- Only healthy and productive palms should be selected as LSU palms (because we are not interested in the nutritional status of abnormal and unproductive palms). Make sure sterile, abnormal, leaning, supply, and diseased palms are not selected as LSU palms.
- All LSU palms are marked, georeferenced and mapped on isometric paper and/or digital maps. Laminated maps can be used in the field to assist with LSU palm identification.
- All LSU palms are labelled with a QR code. The sampling team 'read' the QR card with a hand held device to verify that the palm has been visited during leaf sampling.
- Record the number of LSU palms in each block and verify that the sampling density is at east 1%:

$$\text{Sampling density} = \frac{\text{Number of LSU palms}}{\text{Density (palm/ha)} \times \text{area(ha)}}$$

3. Selection of reference leaf tissue

Leaves on the oil palm are arranged in eight parastichies and each parastichy contains 5–8 leaves (Figure 2). The first fully open leaf is Leaf 1 and the 6th leaf in the 1st parastichy is Leaf 41. The reference leaf for leaf analysis has been nominated based on a thorough analysis of the physiological characteristics of the oil palm leaf canopy:

- Leaf 9 is used in immature palms <3 YAP
- Leaf 17 (located in the middle of the leaf canopy) is used in mature palms ≥3 YAP.

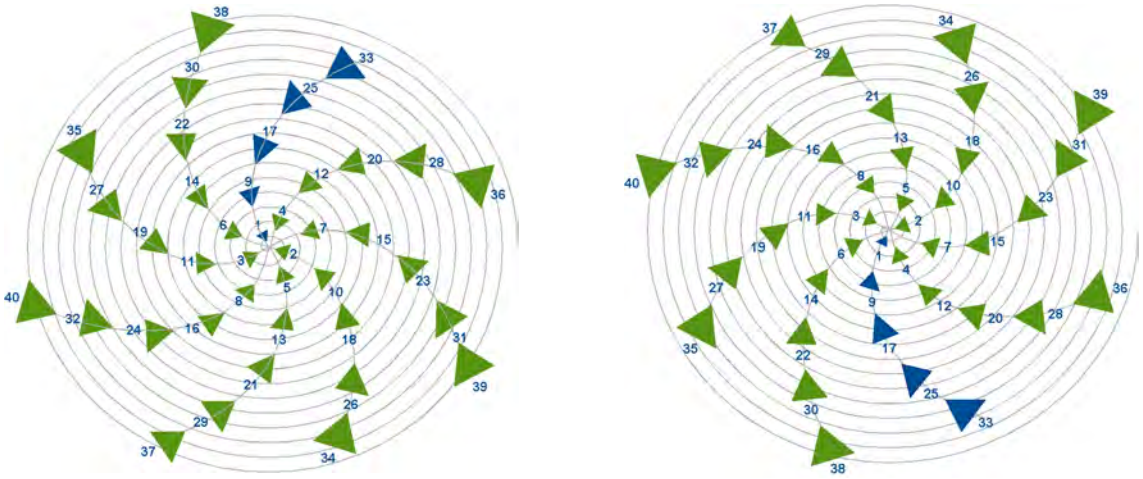

Figure 2. Phyllotaxis in left-handed (left) and right-handed (right) palms. Leaves in one spiral (e.g., Leaf 1, 9, 17, 25, 33, and 41) constitute one parastichy.

Because LSU palms are marked, it is easy for a supervisor to check that the correct leaf has been sampled by spot-checking a few LSU palms in several blocks immediately after leaf sampling has been completed.

Mature palms produce about 1.5–2.5 leaves per month or 18–30 leaves per year and therefore Leaf 17 is 7–11 months old and Leaf 9 is 4–6 months old.

The reference leaf pinnae tissue on the reference leaf is '12 leaflets per leaf (i.e., 6 leaflets (3 upper and 3 lower rank) from each side of a point on the rachis ⅔rds of the distance form the insertion point of the first true leaves and the leaf tip'. The correct position coincides with the point where the ridge on the upper surface of the rachis tapers to a point (Photo 1).

Incorrect identification of reference leaf tissue leads to 'false positives' and 'false negatives' concerning leaf nutrient deficiencies. For example, leaf N, P and K content decreases whilst leaf Ca content increases with leaf age, which has important consequences:

- If the leaf selected is *younger* than the nominated reference leaf, the leaf content of N, P, K and Mg will be *greater*, leading to a false conclusion that palm nutrient status is *sufficient*.
- If the leaf selected is *older* than the nominated reference leaf, the leaf content of N, P, K, and Mg will be *smaller*, leading to a false conclusion that palm nutrient status is *deficient*.

Leaf nutrient content also varies within leaf pinnae. It is therefore important to select the reference tissue of each leaf (i.e., the middle third portion of each leaflet). The leaf pinnae midrib should also be removed and discarded.

Accurate and rapid identification of the reference leaf (Leaf 9 or Leaf 17) requires practice. It is recommended that the agronomist attach a label to all leaves in the palm canopy on a young mature palm (<8 YAP) in an accessible location. Numbered plastic tags (cut out of a used herbicide container)

are attached to the respective leaf rachis with a rubber band (Photo 2). Leaf number changes as the palm grows and therefore the labels should be 'moved' every two months.

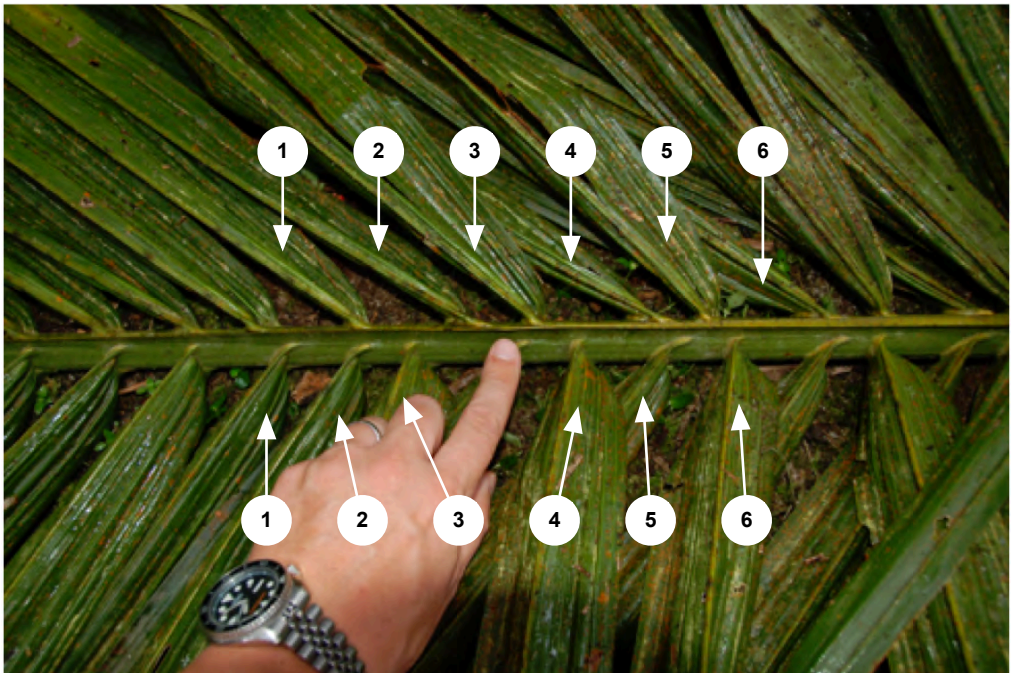

Photo 1. Leaflets are sampled from each side of a point on the rachis ⅔rds of the distance form the insertion point of the first true leaves and the leaf tip are selected for leaf analysis.

Photo 2. All leaf positions have been labeled on a young palm and the relative position of Leaf 1, 9, 17, 25, 33 and 41 is revealed.

This exercise helps to ensure that all personnel know how to identify Leaf 9 and Leaf 17 (the reference leaves for leaf sampling) and Leaf 41 (the leaf subtending the next ripe bunch, used for measuring palm height). Labeling all leaves in the palm canopy also helps agronomy and management staff to understand pruning standards (i.e., number of leaves and leaf parastichies required in relation to palm age) and how to identify the present position of Leaf 1 identified at the previous leaf sampling exercise.

A refresher course on leaf sampling procedures should be carried out each year before the start of leaf sampling.

4. Sample preparation

All leaf samples should be dried and ground at the plantation. Otherwise it is impossible to disguise cross check samples within a batch of commercial samples.

Some steps in sample preparation affect leaf nutrient content:

- Samples must be dried <24 hours after sampling. Leaf N content is affected if leaf drying is delayed.
- Leaf samples should be dried in a forced draft oven at 70–80 ^{o}C. Leaf N content is reduced if leaves are dried at >80 ^{o}C.
- Dried samples are ground to pass a 18-mesh (or 1 mm) sieve using e.g., a Thomas Wiley mill or a Tecator Cyclotec grinder. Uniform particle size is important because a very small amount of sample (±0.5 g) from the block sample (600 g) is used for analysis (Figure 3).

A very small amount of sample (0.2–0.5 g) is used for each analysis. Therefore, make sure that the sample sent to the laboratory (±50 g) is representative of the block sample (±600 g) (Figure 3).

To prepare a representative sub-sample:

- Thoroughly mix the block sample and place it on a clean plastic sheet.
- Form the sample into a cone and divide the cone into four quadrants.
- Use a 5 ml laboratory spoon and take one spoonful from each quadrant of the cone in turn until the plastic sample bag contains 50 g of sample material.

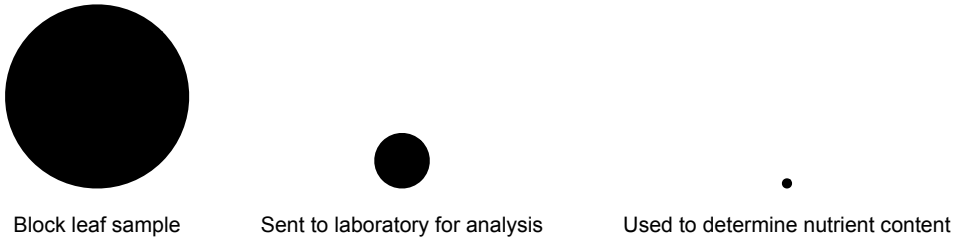

Figure 3. Relative size of a block leaf sample (600 g), sample sent to the laboratory (50 g) and sample used for a nutrient determination (0.5 g)

Samples are packed in labelled plastic bags before dispatch to the lab.

5. Analysis at a service laboratory

Laboratory cross checking is essential, even if the laboratory is owned by the plantation company, whenever the results of leaf analysis are used to determine palm nutrient status for the purpose of making fertilizer recommendations.

The key issues for accurate and precise laboratory work are:

- Well-maintained equipment.
- High quality reagents.

- Skilled, experienced and diligent analysts.

These aspects of laboratory management are, of course, outside the supervision of the plantation company. However, an observant representative of the plantation can get a fairly clear impression of a service laboratory during a visit. As a general rule, a busy laboratory will provide better results because the analysts are practising their skills each day.

5.1. WEPAL participation

The selected service laboratory should be a participant in the Wageningen Evaluating Programme for Analytical Laboratories (WEPAL), run by the University of Wageningen. WEPAL requires member laboratories to analyze blind samples with acceptable levels of accuracy and precision in order to maintain accreditation. However, WEPAL only verifies whether or not a laboratory is *capable* of accurate and precise analysis – **it does not necessarily mean that the laboratory will perform accurate and precise analysis on a set of commercial samples!**

5.2. Cross checking for errors

Errors in laboratory analysis can lead to significant and costly mistakes in fertilizer recommendations. Therefore, whilst a service laboratory may run their own internal quality control cross checks, and participate in the WEPAL certification scheme, the plantation should run independent 'external' cross checks on the service lab to verify, with each batch of commercial samples, that analytical work meets the required standards.

It is important to assess both accuracy and precision of laboratory analysis:

- Analysis is *accurate* when the value reported does not differ from the 'true value'. Accuracy is the nearness of a measurement or the mean of a set of measurements to the true value (i.e., the known accepted value of a quantifiable property).
- Analysis is *precise* when multiple analyses of a given sample agree with each other. Precision is sometimes referred to as 'reproducibility' and is a measure of the random error associated with a series of repeated measurements of the same parameter within a sample.

Precision and accuracy are specific to the analyst and instrument and checks are therefore required when the analyst is changed or new instruments are installed.

Examples of accuracy and precision are illustrated in Figure 4.

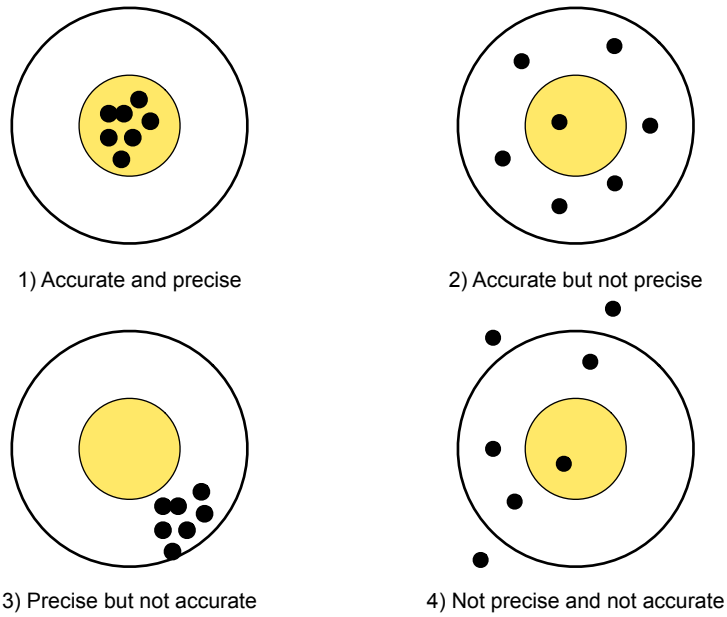

Figure 4. Examples of precision and accuracy in the analysis of seven identical samples.

Results may be:

- ‘Accurate and precise’ (i.e., small variation between seven identical samples, results close to ‘true value’, Figure 1.1).
- ‘Accurate but not precise’ (i.e., large variation between seven identical samples, results close to ‘true value’ (Figure 1.2).
- ‘Precise but not accurate’ (i.e., small variation between identical samples but results different from ‘true value’, Figure 1.3).
- ‘Not precise and not accurate’ (i.e., large variation between identical samples and results different from ‘true value’, Figure 1.4).

We will now detail methods to assess laboratory accuracy and precision.

5.2.1. Assessment of accuracy

To assess accuracy, the plantation must prepare a standard leaf sample (SLS) with known ‘true values’ for the concentration of all nutrients (N, P, K, Mg, Ca, B, Cl, Mn, Zn). The SLS can be prepared by sampling Leaf 17 on 1,000 palms in the usual way. Samples are then dried, ground (1 mm or 18 mesh sieve) and thoroughly mixed to produce a uniform and homogenous sample of ground material. About 5 kg of SLS should be prepared since the standard sample should be sufficient for accuracy crosschecking exercises carried out over 5–10 years (7 samples @ 0.05 kg per year = 0.35 kg/year). The standard sample is stored in an airtight glass container.
The ‘true value’ of this sample can be determined by analysis at a highly reputed laboratory. Seven identical samples are prepared from the standard sample and sent for analysis (N, P, K, Mg, B, Cu, Zn, Mn) at a reliable benchmark laboratory (e.g., UC Davis, WEPAL, Rothamsted). The mean value for each nutrient can be used as the ‘true value’ for the standard sample when assessing the accuracy of service laboratories.

The minimum detection level (MDL) provides an indication of the minimum amount of analyte that the lab can detect. MDL is calculated as follows:

$$MDL = t_{(6,0.01\%)} \times S$$

Where S is the standard deviation for the seven samples. The t value for 99% and 6 degrees of freedom (n=7, 7-1=6) is 3.143.

In a hypothetical example, seven samples were sent to a research laboratory to determine ‘true value’ for the N content of the SLS. The mean value was 2.50 ±0.016 % N or 2.49–2.52% N and the MDL was 0.05 (Table 2).

An identical set of seven samples was sent to a candidate service laboratory to check on accuracy. The mean value was 2.62 ±0.057 % N or 2.57–2.68% N and the MDL was 0.19 (Table 2). Based on this assessment the candidate service laboratory was rejected because the analysis was insufficiently accurate.

Results can be plotted in a graph showing the mean value and confidence interval for the seven standard leaf samples (SLS) and the ‘true value’ for the standard sample (Figure Xa). In this example, service laboratory performance was good in 2012. The mean from the seven SLS was close to the ‘true value’, indicating good accuracy and the calculated confidence interval was small, indicating good precision. In 2013 the mean from the SLS was also close to the true value but variability amongst the seven SLS was high, indicating acceptable accuracy but lack of precision, In 2014 the mean value for the SLS was greater than the ‘true value’, indicating lack of accuracy but the confidence intervals were small indicating acceptable precision. In 2015 both accuracy and precision was poor.

Table 2. Hypothetical example of analysis of standard leaf material and the calculation of the minimum detection level.

Sample	#	Formula	Leaf N%	
			Research lab	Service lab
1			2.50	2.63
2			2.52	2.52
3			2.48	2.68
4			2.49	2.70
5			2.51	2.58
6			2.50	2.61
7			2.53	2.65
Number of samples (n)	a	=COUNT(S1:S9)	7.00	7.00
Mean (x)	b	=AVERAGE(S1:S9)	2.50	2.62
Standard deviation (S)	c	=STDEV.S(S1:S9)	0.0172	0.0613
Sample variance (S^2)	d	c^2	0.0003	0.0038
Standard error (SE)	e	=√d/a-1	0.006	0.023
t-value ($t_{(6,2.5\%)}$)	f	t(6,2.5%)	2.447	2.447
Confidence interval ($CI_{(95\%)}$)	g	e x f	0.016	0.057
Upper limit	h	b + g	2.52	2.68
Lower limit	i	b - g	2.49	2.57
Minimum detection limit (MDL)	j	3.143 x c	0.05	0.19
Maximum relative percent difference (RPD max)	k		2	7

5.2.2. Assessment of precision

Precision can be assessed by measuring the difference between the values obtained for pairs of identical samples. For this purpose, five paired samples are sent for analysis. The paired samples can be prepared by dividing five commercial samples into two identical sub-samples. The five commercial samples selected for this exercise should represent a rage of nutrient status (i.e., from deficient blocks to blocks with good nutritional status, based on past analysis) so that precision can be assessed at small and large nutrient concentrations.

An estimate of precision is provided by the relative percent difference (RPD). The relative percent difference (RPD) is calculated as follows:

$$\text{Relative percent difference} = \left(\frac{a-b}{(a+b)/2} \right) x100$$

Where *a* and *b* are a sample pair.

For example, if sample a = 2.50% N and sample b = 2.45% N,

$$\text{RPD} = \left(\frac{2.50-2.45}{(2.50+2.45)/2} \right) x100$$

$$= \frac{0.05}{2.48} x100 = 2\%$$

Lower values for RPD indicate greater precision and, for most analysis, the RPD should be <5%. Really good laboratories achieve RPD of <2%.

The standard deviation of *differences* between paired samples provides an indication of precision in the batch of five paired samples:

$$S=\sqrt{\frac{\sum_{i=1}^{n}\left(x_1-x_2\right)_i^2}{2n}}$$

Where S = standard deviation of the difference between duplicate pairs, N = number of duplicate pairs and $(x_1-x_2)_i$ = difference of the *i*th pair. The greater the value of the standard deviation of the difference between duplicate pairs, the poorer the precision in analysis.

In a hypothetical example, we can compare the RPD for five paired samples analyzed in two laboratories (Table 3). In this example, the RPD values for Lab 1 ranged from 1–2, indicating good precision and the standard deviation of differences was 0.20. By contrast, in Lab 2 the RPD values range from 3–16, indicating poor precision and the standard deviation of differences was 0.204

Table 3. Results for the analysis of five paired samples in two service laboratories.

Sample	Lab 1				Lab 2			
	A	B	RPD	$(a-b)^2$	A	B	RPD	$(a-b)^2$
S1	2.45	2.49	2	0.0016	2.45	2.53	3	0.0064
S2	2.35	2.40	2	0.0025	2.50	2.30	8	0.0400
S3	2.52	2.54	1	0.0004	2.70	3.00	11	0.0900
S4	2.86	2.79	2	0.0049	2.20	2.40	9	0.0400
S5	3.15	3.20	2	0.0025	3.30	2.80	16	0.2500
Average	2.67	2.68	2	0.0024	2.63	2.61	9	0.0853
Standard deviation	0.332	0.323		0.2041	0.415	0.289		1.2216

We can conclude that in our hypothetical example, Lab 1 provides a high degree of precision whilst the precision of Lab 2 is insufficient.

Results from the two laboratories can be plotted in a graph to show the degree of precision between paired samples (Figure 5b). The results for Lab 1 line up closely to a 1:1 line, indicating good precision at all concentrations of N. The results for Lab 2 are poorly aligned to a 1:1 line, particularly at high concentrations of N, indicating poor precision.

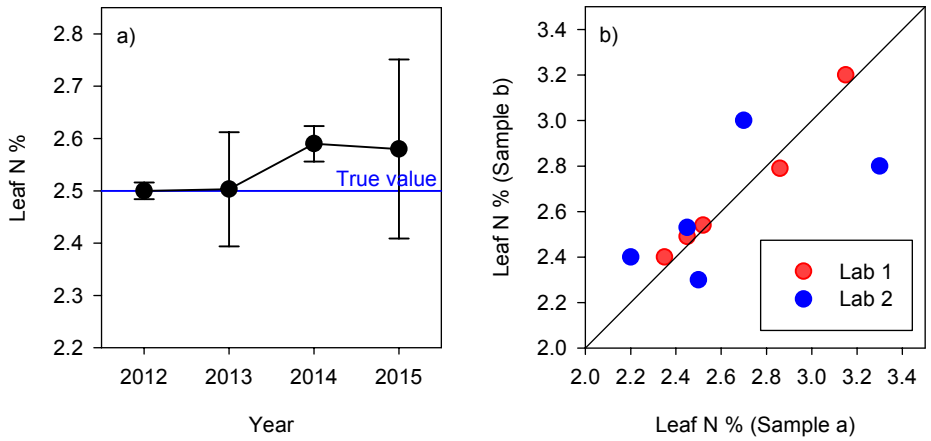

Figure 5. Graphs showing mean N concentration for a standard leaf sample over a four-year period (Bars represent 95% confidence intervals and the 'true value' is shown in blue) (a) and precision between five pairs of samples analyzed for leaf N content at two laboratories (b).

5.3. Cross check sample labelling

It is important that the laboratory is not provided with any information on the provenance (e.g., block ID, palm age, planting material, cross check sample) of samples. Instead, each sample is labelled with a six-digit ID code (e.g., 010123) and details of sample provenance re stored with the sample ID code by the plantation in a spreadsheet. It is particularly important to tag all crosscheck samples with a label similar in format and appearance to commercial samples.

It is always best to include all cross check samples with routine commercial samples so that it is impossible for the analyst to guess the provenance of the samples. The laboratory should be informed, however, that each set of commercial samples contains a set of cross check samples to assess laboratory accuracy and precision. This will encourage the laboratory to carry out the analytical work diligently.

5.4. Assessment of laboratory

Accuracy and precision is greater for some assays (e.g., RPD ±2% for leaf N content measured in a C/N auto analyzer) than others (e.g., ±5% for leaf P content measured by colorimetric method). Therefore, assessments for accuracy and precision should be carried out for all nutrients (N, P, K, Mg, Ca, B, Cu, Zn, Mn).

It may also be worthwhile to prepare a standard rachis sample (particularly since the content of N and P is much lower and K much higher than in leaf samples) and a standard soil sample.

The assessment of accuracy and precision should be carried out each year and the results maintained in a computer database. In this way, accuracy and precision can be monitored conveniently over time.

6. Data processing

6.1. Correction of commercial leaf analysis data for seasonal variation

In most locations, leaf nutrient content varies through the year. For example, the concentration of nutrients in samples taken at the end of the dry season is often notably smaller than in samples taken at the end of the wet season. Data from annual leaf sampling should therefore be corrected for seasonal fluctuations. Carry out leaf sampling in X % of blocks every two months (Table X).

Table 4. Results from bimonthly sampling for leaf N

Survey block	Leaf N
February	2.51
April	2.41
June	2.48
August	2.57
October	2.61
December	2.53
Average	2.52

To correct data from commercial blocks, add the difference between the mean value and the value for the month closest to the actual sampling month for the commercial block. For example, if the commercial sample value for leaf N sampled in October was 2.48 %N, the corrected value is calculated as follows.

$$\text{Corrected leaf N\%} = 2.48 + (2.61 - 2.52) = 2.48 + 0.09 = 2.57$$

All leaf and rachis data should be corrected for seasonal variation.

6.2. Transpositional errors

Transpositional errors often arise when data is transferred from laboratory reports to the company agronomic database. To reduce the chance of error:

- Use two operators to enter data (i.e., one to read out the data and the other to enter the data in the computer).
- Implement double data entry (i.e., the same value must be entered twice) in the company agronomic database.

7. Conclusions

- A total of 17 samples (5 paired samples and 7 standard leaf samples) will provide a high degree of reassurance concerning the accuracy and precision of a particular service laboratory.
  - An alternative laboratory should be sought where accuracy and precision are found to be poor.
  - Where high levels of accuracy and precision are evident, the agronomist can be confident that the results from leaf sampling and analysis can be interpreted rigorously for the purpose of estimating fertilizer requirements.
- A cross checking exercise should be carried out with a highly reputed research laboratory to determine 'true values' for each analyte and repeated each year with the service laboratory, using results from the research laboratory as a benchmark.
- The WEPAL cross checking system only verifies whether or not a laboratory is *capable* of carrying out accurate analytical work. A laboratory certified by WEPAL might not necessarily deliver accurate and precise results for samples presented by a commercial company.
- Always carry out cross checks for accuracy and precision before selecting a service laboratory.
- Carry out cross checks for accuracy and precision each year to verify that the selected laboratory has maintained standards. Both accuracy and precision may change with changes in equipment, personnel
- Prepare a standard sample of leaf (and rachis) material. Send seven identical samples of the sample to a highly reputed laboratory to determine the 'true value' for nutrient content (N, P, K, Mg, Ca, B, Cu, Zn).
- Always inform the lab that commercial samples include indistinguishable crosscheck samples. This will encourage the laboratory to take more care with the analytical work.
- Keep a record of all assessments of accuracy and precision for the purpose of comparison and to track accuracy and precision over time.
- Avoid the temptation to attribute significance to the results of leaf analysis greater than the accuracy and precision indicated by cross checking exercises.

Table 5. Summary of the main causes of error in laboratory analysis

Activity	Source of error
LSU blocks	When blocks are grouped in leaf sampling units (LSUs) the sample block may not be representative of the other blocks in the LSU.
LSU palm selection	Unproductive or abnormal palms selected as LSU. Sampling density (LSU palms/ha) insufficient. LSU palms not distributed evenly within the sample block.
Leaf and rachis sampling	Incorrect leaf sampled. Samples mislabeled.
Sample preparation	Oven temperature too high (>80 ^{o}C). Leaves contaminated by unnecessary and/or ineffective washing before drying. Samples mislabeled.

Laboratory analysis	Insufficient internal quality assurance programmes Incorrect method used. Procedures not followed correctly. Faulty or poorly maintained equipment. Poor calibration of equipment and standards. Poor quality or contaminated reagents.
Data processing	Data entered incorrectly. Confusion over labelling leads to data entry errors.

Table 6. Acronyms

Term	Definition
YAP	Year after planting
LSU	Leaf sampling unit. A set of blocks so similar in terms of palm age, soil type, planting material and agronomic conditions that they may be considered as a single unit for the purpose of fertilizer recommendations.
YAP	Years after planting. The age of the palm (calculated as the present year minus year of planting)
WEPAL	Wageningen Evaluating Programme for Analytical Laboratories
MDL	Minimum detection level
CI	Confidence interval
SE	Standard error
S	Standard deviation
S^2	Sample variance
t	Value from table of t-values
RPD	Relative percent difference