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Editors:

Roy Hendroko Setyobudi, Hugo Scheer, Leenawaty Limantara, Yuzo Shioi, Leszek Fiedor, Tatas H.P. Brotosudarmo and Monika N.U. Prihastyanti

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Analysis on the Chlorophyll Content of Commercial Green Leafy Vegetables

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Abstract

The objectives of the present study were to evaluate the chlorophyll content of green leafy vegetables found commercially and carry out a comparative investigation between in vivo and in vitro data. The chlorophyll of green leafy vegetable can be used as visible parameters of the quality of vegetables during storage, since it will be degraded gradually along with post-harvest senescence. Therefore, the development of reliable in vivo chlorophyll measurement should be advantageous rather than visual observation for the purpose of quality control and product sortation. Here, the existence of chlorophylls in ten green leafy vegetables were reported as SPAD values of a handheld SPAD-502 chlorophyll meter and % N of an Agriexpert CCN-6000 nitrogen meter (in vivo data), as well as total peak area data of HPLC measurement for chlorophyll *a* and *b* after exhaustive extraction using methanol (in vitro data). Both in vivo and in vitro measurement gave comparable grouping of vegetables with high and low content of chlorophyll. Moreover, correlation plots between SPAD values and total peak area of HPLC showed adequate linear correlation ($R^2 > 0.7$), revealing the potency of in vivo observation for the prediction of actual chlorophyll content in commercial leafy vegetables. SPAD values and % N presented strong linear relationship ($R^2 > 0.9$), in which SPAD-meter performed better detection at very low values. The calibration curve for each species of vegetable should be substantial to overcome the limiting factors of in vivo observation, such as leaf size, tissue thickness, and variation of chloroplast distribution.

Peer-review under responsibility of the Scientific Committee of HK-ICONS 2014

Keywords: Chlorophyll; HPLC; leafy vegetable; nitrogen meter; SPAD

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Nomenclature

SPAD	Soil Plant Analysis Development
HPLC	High Performance Liquid Chromatography
R^2	coefficient of determination or goodness-of-fit of linear regression
sp.	species
subsp.	subspecies
var.	variety
s	second (60 s = 1 min; 60 min = 1 h)

1. Introduction

Nowadays, consumption of green leafy vegetables has been increasing, especially as a counterbalance of the growing number of degenerative diseases. Several bioactive compounds in vegetables are including vitamins, minerals, antioxidants, as well as the pigments (chlorophylls and carotenoids). In the living plants, chlorophylls play an important role as primary photosynthetic pigment to capture light energy from the sun. Composed together with carotenoids (the accessory photosynthetic pigments) in pigment-protein complexes, it exhibits colour appearance which is specific for each plant leaf and even used as parameter of maturity, quality, and freshness of food crops[1,2]. The colour is extremely important because it defines the appearance of the vegetables and influences consumer choice[3]. However, this minimally processed vegetables are most kept under low temperature preservation (usually 4 °C to 10 °C, with 95 % to 100 % relative humidity) and sold within one week. During that period, the physiological processes occur and particularly cause loss of colour due to degradation of leaf pigments or tissue browning. Hence the quality and freshness of commercially sold vegetable can be monitored by measuring its chlorophyll contents[4].

The content of chlorophylls can be determined photometrically following extraction of the pigments using an organic solvent, such as acetone or dimethyl formamide, or else by means of handheld device based on light-emitting diodes and silicon photodiode receptor that measures leaf transmittance in the red (650 nm) and infrared (950 nm) regions of the electromagnetic spectrum[5]. The transmittance values are used by the device to derive a relative SPAD meter values that is proportional to the amount of chlorophyll in the sample. The former method is considered as in vitro measurement which is well established and accurate, but time-consuming, destructive, and requires the use of toxic or flammable chemicals. On the other hand, the latter provides an alternative in vivo method for the measurements of relative leaf chlorophyll levels that overcome these disadvantages, but it is less accurate, not applicable for small or thick leaf, influenced by light condition, and produces only predictive value.

There have been numerous studies which evaluated the correlation between in vitro chlorophyll data with its in vivo data, based on SPAD value as well percentage of nitrogen in leaf, by using available handheld instruments. The correlation between SPAD value and % N was mostly found as strong linear function, while the strong relationship ($R^2 \sim 0.9$) between SPAD value and in vitro chlorophyll concentration has been previously proposed to follow exponential[6] or second-order polynomial function[5,7]. Most of the studies employed the leaves of growing plant at certain medium or light set up, but there are only a few numbers of investigations which employed post-harvested samples. Here, the correlation between in vivo and in vitro data of the chlorophyll content of ten green leafy vegetables purchased from three different supermarkets at Malang, East Java, Indonesia, was observed. The objectives of this study were to (i) identify the range of the SPAD value and % N for ten selected green leafy vegetables on their condition in the market; and (ii) find out the distribution of the feasibility of in vivo measurement among ten selected leafy vegetables, compared to their in vitro data.

2. Materials and methods

2.1. Plant materials

Green leafy vegetables were collected from three different supermarkets in Malang area (East Java, Indonesia). All samples were purchased early in the morning, transported into laboratory and directly measured by in vivo procedure for SPAD value and % N. Then, the samples were stored at -18 °C deep freezer for further in vitro analysis. The whole experiment was accomplished during May 2014 until June 2014.

2.2. In vivo assay

The relative chlorophyll content (SPAD value) and % N were determined by means of SPAD-502 chlorophyll meter (Konica Minolta, Japan) and nitrogen meter Agriexpert CCN 6000 (Satake, Japan), respectively. The adaxial side of the leaves was always placed toward the emitting window of the instrument and major veins were avoided. Each leaf, based on its total leaf area, was marked in five up to 14 representative points for in vivo measurements, and the average value was calculated. As for basil, due to its small size, seven leaves were used with two up to three spots of measurement each. This method was intended to overcome the influence of non-uniform distribution of chlorophyll in the leaf, and hence producing more representative data.

2.3. In vitro assay

Liquid nitrogen was poured to accurately weighted 0.2 g of leaf samples, and then the frozen leaf was ground in a porcelain mortar and added with 3 mL methanol (Merck, Germany). The extraction was continued by using Vortex for 10 s with inert atmosphere. After that, the extract was filtered (0.22 µm) and injected to HPLC instrument. Each sample was injected to the HPLC in triplicate. All steps were carried out under dim light to prevent any degradation of photosynthetic pigments. The chromatography was performed on a Shimadzu LC20AD system (Shimadzu, Japan) equipped with photo diode array detector and CTO 20A column oven. LC Solution chromatographic software (Shimadzu, Japan) was used for data processing and evaluation. The RP-18e column (Chromolith, Germany) was used for separation, using methanol (Merck, Germany) and acetonitrile (Merck, Germany) of 50 : 50 (v/v) as mobile phase. The flow rate was adjusted to 0.5 mL · min^{-1} and detection was performed over the range 400 nm to 700 nm.

2.4. Data analysis

The figures were created from numeric data and plotted using Origin 7 Software (OriginLab) and Microsoft Excel 2013. The data were expressed as the mean ± standard error of the mean (SEM).

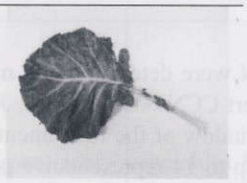
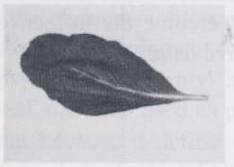
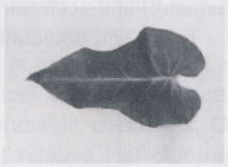
3. Results and discussion

Ten green leafy vegetables were successfully collected from three different supermarkets in Malang (Table 1), except basil and sword-leaf lettuce which were not available in the third supermarket during this time of research. After purchasing, samples were subsequently subjected to in vivo and in vitro measurement. All measurements were conducted under the same light condition in order to eliminate data variation caused by difference of light interference which may lead to pigment degradation or any disturbance in instrumentation. Identical in vitro extraction and HPLC procedure was similarly employed for all samples.

Fig. 1 shows the SPAD values of ten green leafy vegetables purchased from three supermarkets in Malang. The average of SPAD values ranges from the lowest 2.7 ± 0.8 of head cabbage (Kbs) up to the highest 52.4 ± 3.7 of Chinese cabbage pak-choi (Pch). The SPAD value is proportional to the relative amount of chlorophyll content and consistent with their colour appearance, in which the head cabbage has light green leaves, whereas Chinese cabbage pak-choi has darker green foliage. Higher SPAD value signifies higher chlorophyll content. Three vegetables showed low SPAD value (below 30), i.e. head cabbage (Kbs), green leaf lettuce (Sel), and sword-leaf lettuce (Sak).

On the other hand, seven other vegetables have SPAD values which are greater, supposing that the vegetables are rich in chlorophyll. The variation of SPAD values among the same species can be influenced by the time of harvest, the nature heterogeneity among species[8], and the difference in growth conditions which may lead to a redistribution of chloroplasts within mesophyll cells[9].

Table 1. The ten species of leafy vegetables collected from three local markets in Malang, East Java, Indonesia.

No	Vegetables (Local name, scientific name)	Pictures	No	Vegetables (Local name, scientific name)	Pictures
1	Chinese kale (Kailan, *Brassica oleracea* var. *alboglabra* Bailey)		6	Green leaf lettuce (Selada, *Lactuca sativa* L.)	
2	Chinese flowering cabbage (Chaisim or sawi hijau, *Brassica rapa* L. var. *parachinensis* (L. H. Bailey) Hanelt		7	Chinese cabbage pak-choi (Pakcoy, *Brassica rapa* L. subsp. *chinensis*)	
3	Spinach (Bayam, *Amaranthus spinosus* L.)		8	Basil (Kemangi, *Ocimum citriodorum* Vis.)	
4	Water spinach (Kangkung, *Ipomoea aquatica* Forsk)		9	Sword-leaf lettuce (Siomak, *Lactuca sativa* L. var. *augustana*)	
5	Cassava leaf (Daun Singkong, *Manihot utilissima* Pohl)		10	Head cabbage (Kubis, *Brassica oleracea* L. var. *capitata*)	

Abbreviation: Chinese kale (Kai), Chinese flowering cabbage (Cha), spinach (Bay), water spinach (Kan), cassava leaf (Sin), green leaf lettuce (Sel), Chinese cabbage pak-choi (Pch), basil (Kgi), sword-leaf lettuce (Sak), head cabbage (Kbs).

Furthermore, nitrogen meter displays a direct value of nitrogen percentage (% N) which presents on the leafy vegetables. The characteristic of nitrogen percentages was comparable to SPAD values, varying from 0.8 % ± 0.1 % for green leaf lettuce (Sel) up to 4.8 % ± 0.4 % for Chinese cabbage pak-choi (Pch) (Fig. 1). Unfortunately, the nitrogen meter value of head cabbage (Kbs) was exempted due to its unmeasurable thick leaf and pale colour. Seven leafy vegetables, composed as the previous discussion of SPAD value, indicated the presence of nitrogen from

ater, supposing that the vegetables are
n be influenced by the time of harvest.
ons which may lead to a redistribution

va, Indonesia.

ables cientific name)	Pictures
ice *ca sativa* L.)	
ge pak-choi *ica rapa* L. *is*)	
mum s.)	
uce *uca sativa* L.)	
ca oleracea L.	

spinach (Kan), cassava leaf (Sin), green leaf
(Sak), head cabbage (Kbs).

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choi (Pch) (Fig. 1). Unfortunately, the
urable thick leaf and pale colour. Seven
dicated the presence of nitrogen from

2.9 % ± 0.2 % up to 4.8 % ± 0 .4 % which is fairly higher than those of head cabbage (Kbs), green leaf lettuce (Sel), and sword-leaf lettuce (Sak) whose values were less than 2.4 % ± 0.2 %.

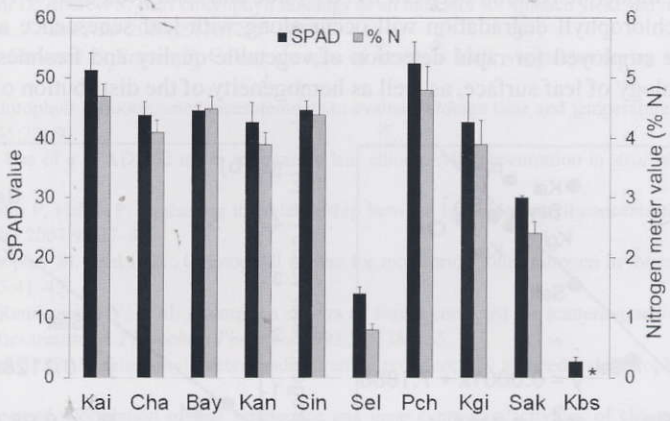

Fig. 1. In vivo analysis of green leafy vegetables by means of SPAD-502 chlorophyll meter and nitrogen meter. Abbreviations were defined in Table 1. *: Head cabbage (Kbs) was unmeasurable by the nitrogen meter.

The measurement by means of chlorophyll and nitrogen meter is based on the principle of detecting absorbance of certain wavelengths which were sent through the leaf tissue. Both systems use particular algorithms for internal calculations in order to produce in vivo data. This method is non-destructive, cheaper, and can be accomplished within a short time after sample collection. However, in vitro quantification is often needed for any verification and comparison purpose. In the present study, HPLC was employed to quantify chlorophyll content of each green leafy vegetable after extraction using organic solvent. Fig. 2 shows data of total peak area, obtained from HPLC measurements, which are proportional to the actual amount of chlorophylls present in the leaf. It was consistent with the result of in vivo measurements using chlorophyll and nitrogen meter, three vegetables showed an average of total peak area < 200 000, i. e. head cabbage (Kbs), green leaf lettuce (Sel), and sword-leaf lettuce (Sak). Nevertheless, the other seven vegetables were not in the same order as observed through in vivo measurement, revealing that in vitro detection is somehow needed for actual data verification.

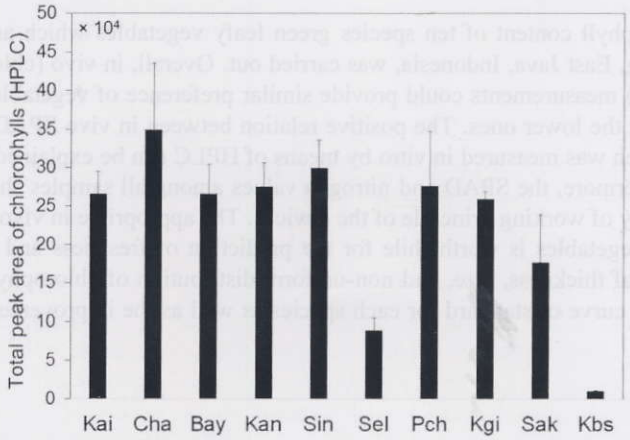

Fig. 2. In vitro analysis of the methanol extract of green leafy vegetables by means of HPLC, expressed as total peak area of chlorophyll *a* and *b* monitored at 400 nm to 700 nm. Abbreviations were defined in Table 1.

In order to find out the distribution of the feasibility of in vivo measurement among ten selected leafy vegetables compared to their in vitro data, the data plot between total peak area of chlorophylls (HPLC) and SPAD value was generated (Fig. 3a). The best-fit regression was obtained as linear correlation with goodness of fit (R^2) > 0.7, revealing the presence of moderate relation between SPAD value and total peak area of HPLC for chlorophyll *a* and *b*. In green vegetables, chlorophyll degradation will occur along with leaf senescence after harvest[3,10]. Therefore, chlorophyll meter can be employed for rapid detection of vegetable quality and freshness. Several limiting factors are the thickness, morphology of leaf surface, as well as homogeneity of the distribution of chlorophyll in leaves.

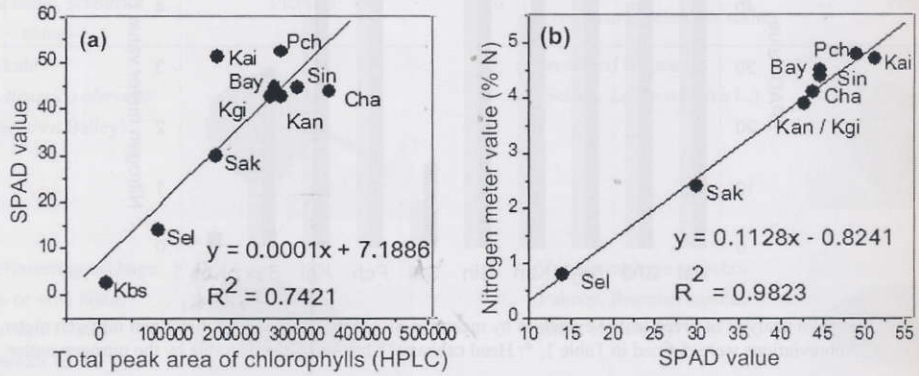

Fig. 3. Data plots of SPAD values versus total peak area of chlorophyll *a* and *b* obtained from HPLC measurement (a), and nitrogen meter versus SPAD values (b). The equation of the linear regression line and its coefficient of determination (R^2-value) is given in the figure. Abbreviations were defined in Table 1.

In addition, appreciable linear fit (R^2 = 0.98) was found in the positive correlation among in vivo measurement (Fig. 3b). This finding is comparable with the result of Johan et al.[6] and Qihua et al.[5], in which a strong linear relationship was observed between SPAD and nitrogen meter values. This is acceptable since the development of nitrogen meter is technically based on the chlorophyll meter (SPAD-502)[6]. However, beside the influence of leaf thickness, the SPAD-502 may be more reliable at very low values, since the lowest SPAD value of Kbs was not recorded by nitrogen meter.

4. Conclusion

Investigation on chlorophyll content of ten species green leafy vegetables which are common in daily diet of local community in Malang, East Java, Indonesia, was carried out. Overall, in vivo (chlorophyll meter and nitrogen meter) and in vitro (HPLC) measurements could provide similar preference of vegetables having significantly high content of chlorophyll than the lower ones. The positive relation between in vivo SPAD values and total peak area of chlorophyll *a* and *b* which was measured in vitro by means of HPLC can be explained as 74 % of the variation in a linear relationship. Furthermore, the SPAD and nitrogen values among all samples showed strong relationship of 98 % based on the similarity of working principle of the devices. The appropriate in vivo detection of photosynthetic pigments on green leafy vegetables is worthwhile for the prediction of freshness and nutritional quality. Several limiting factors, such as leaf thickness, size, and non-uniform distribution of chlorophyll, can be overcome by the development of calibration curve of standard for each species as well as the improvement of measuring capacity of the instrumentation.

Acknowledgements

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References

1. Qibing W, Jianjun C, Rober
Nutr 2005;28(7):1215–1225
2. Yan JL, Yi PT, Yong GZ, H
nitrogen fertilizer applicatio
3. Antonio F, Luca I, Rita M
2004;2(3&4):40–44.
4. Antonio F, Tommaso M. C
Postharvest Biol Tech 2007
5. Qihua L, Weihua H, Paul J
2011;107(2):209–214.
6. Johan UF, Johanna GA, Kr
meter readings. *Photosynth*
7. Dilfuza D, John PAL, Chri
Asia. *J Arid Environ* 2012;
8. Fukshansky L, Martinez A,
transfer and absorption stati
9. Jan N, Jitka P, Jiri R, Mart
2010;105(3):265–271.
10. Xuelian Z, Zhaoqi Z, Jin
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References

1. Qibing W, Jianjun C, Robert HS, Yuncong L. Correlation of visual quality grading and SPAD reading of green-leaved foliage plants. *J Plant Nutr* 2005;28(7):1215–1225.
2. Yan JL, Yi PT, Yong GZ, Hui D, Andrew S. Leaf chlorophyll readings as an indicator for spinach yield and nutritional quality with different nitrogen fertilizer applications. *J Plant Nutr* 2006;29(7):1207–1217.
3. Antonio F, Luca I, Rita M, Giovanni S, Franco T. Colour changes of fresh-cut leafy vegetables during storage. *J Food Agric Environ* 2004;2(3&4):40–44.
4. Antonio F, Tommaso M. Chlorophyll *a* fluorescence measurements to evaluate storage time and temperature of *Valeriana* leafy vegetables. *Postharvest Biol Tech* 2007;45:73–80.
5. Qihua L, Weihua H, Paul J. Use of a SPAD-502 meter to measure leaf chlorophyll concentration in *Arabidopsis thaliana*. *Photosynth Res* 2011;107(2):209–214.
6. Johan UF, Johanna GA, Kristin P, Hakan P. Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. *Photosynth Res* 2007;91:37–46.
7. Dilfuza D, John PAL, Christopher M, Paul LGV. Chlorophyll meters for monitoring foliar nitrogen in three tree species from arid Central Asia. *J Arid Environ* 2012; 85:41–45.
8. Fukshansky L, Martinez A, Remisowsky V, et al. Absorption spectra of leaves corrected for scattering and distributional error: a radiative transfer and absorption statistics treatment. *Photochem Photobiol* 1993;57:538–555.
9. Jan N, Jitka P, Jiri R, Martina S. SPAD chlorophyll meter reading can be pronouncedly affected by chloroplast movement. *Photosynth Res* 2010;105(3):265–271.
10. Xuelian Z, Zhaoqi Z, Jin L, et al. Correlation of leaf senescence and gene expression/activities of chlorophyll degradation enzymes in harvested Chinese flowering cabbage (*Brassica rapa* var. parachinensis). *J Plant Physiol* 2011;168(11):2081–2087.

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The relative chlorophyll content (SPAD value) and % N were determined by means of SPAD-502 chlorophyll meter (Konica Minolta, Japan) and nitrogen meter Agriexpert CCN 6000 (Satake, Japan), respectively. The adaxial side of the leaves was always placed toward the emitting window of the instrument and major veins were avoided. Each leaf, based on its total leaf area, was marked in five up to 14 representative points for in vivo measurements, and the average value was calculated. As for basil, due to its small size, seven leaves were used with two up to three spots of measurement each. This method was intended to overcome the influence of non-uniform distribution of chlorophyll in the leaf, and hence producing more representative data.

2.3. In vitro assay

Liquid nitrogen was poured to accurately weighted 0.2 g of leaf samples, and then the frozen leaf was ground in a porcelain mortar and added with 3 mL methanol (Merck, Germany). The extraction was continued by using Vortex for 10 s with inert atmosphere. After that, the extract was filtered (0.22 μm) and injected to HPLC instrument. Each sample was injected to the HPLC in triplicate. All steps were carried out under dim light to prevent any degradation of photosynthetic pigments. The chromatography was performed on a Shimadzu LC20AD system (Shimadzu, Japan) equipped with photo diode array detector and CTO 20A column oven. LC Solution chromatographic software (Shimadzu, Japan) was used for data processing and evaluation. The RP-18e column (Chromolith, Germany) was used for separation, using methanol (Merck, Germany) and acetonitrile (Merck, Germany) of 50 : 50 (v/v) as mobile phase. The flow rate was adjusted to 0.5 mL $\cdot$ min^{-1} and detection was performed over the range 400 nm to 700 nm.

2.4. Data analysis

The figures were created from numeric data and plotted using Origin 7 Software (OriginLab) and Microsoft Excel 2013. The data were expressed as the mean ± standard error of the mean (SEM).

3. Results and discussion

Ten green leafy vegetables were successfully collected from three different supermarkets in Malang (Table 1), except basil and sword-leaf lettuce which were not available in the third supermarket during this time of research. After purchasing, samples were subsequently subjected to in vivo and in vitro measurement. All measurements were conducted under the same light condition in order to eliminate data variation caused by difference of light interference which may lead to pigment degradation or any disturbance in instrumentation. Identical in vitro extraction and HPLC procedure was similarly employed for all samples.

Fig. 1 shows the SPAD values of ten green leafy vegetables purchased from three supermarkets in Malang. The average of SPAD values ranges from the lowest 2.7 ± 0.8 of head cabbage (Kbs) up to the highest 52.4 ± 3.7 of Chinese cabbage pak-choi (Pch). The SPAD value is proportional to the relative amount of chlorophyll content and consistent with their colour appearance, in which the head cabbage has light green leaves, whereas Chinese cabbage pak-choi has darker green foliage. Higher SPAD value signifies higher chlorophyll content. Three vegetables showed low SPAD value (below 30), i.e. head cabbage (Kbs), green leaf lettuce (Sel), and sword-leaf lettuce (Sak).

On the other hand, seven other vegetables have SPAD values which are greater, supposing that the vegetables are rich in chlorophyll. The variation of SPAD values among the same species can be influenced by the time of harvest, the nature heterogeneity among species[8], and the difference in growth conditions which may lead to a redistribution of chloroplasts within mesophyll cells[9].

Table 1. The ten species of leafy vegetables collected from three local markets in Malang, East Java, Indonesia.

No	Vegetables (Local name, scientific name)	Pictures	No	Vegetables (Local name, scientific name)	Pictures
1	Chinese kale (Kailan, *Brassica oleracea* var. *alboglabra* Bailey)		6	Green leaf lettuce (Selada, *Lactuca sativa* L.)	
2	Chinese flowering cabbage (Chaisim or sawi hijau, *Brassica rapa* L. var. *parachinensis* (L. H. Bailey) Hanelt		7	Chinese cabbage pak-choi (Pakcoy, *Brassica rapa* L. subsp. *chinensis*)	
3	Spinach (Bayam, *Amaranthus spinosus* L.)		8	Basil (Kemangi, *Ocimum citriodorum* Vis.)	
4	Water spinach (Kangkung, *Ipomoea aquatica* Forsk)		9	Sword-leaf lettuce (Siomak, *Lactuca sativa* L. var. *augustana*)	
5	Cassava leaf (Daun Singkong, *Manihot utilissima* Pohl)		10	Head cabbage (Kubis, *Brassica oleracea* L. var. *capitata*)	

Abbreviation: Chinese kale (Kai), Chinese flowering cabbage (Cha), spinach (Bay), water spinach (Kan), cassava leaf (Sin), green leaf lettuce (Sel), Chinese cabbage pak-choi (Pch), basil (Kgi), sword-leaf lettuce (Sak), head cabbage (Kbs).

Furthermore, nitrogen meter displays a direct value of nitrogen percentage (% N) which presents on the leafy vegetables. The characteristic of nitrogen percentages was comparable to SPAD values, varying from 0.8 % ± 0.1 % for green leaf lettuce (Sel) up to 4.8 % ± 0.4 % for Chinese cabbage pak-choi (Pch) (Fig. 1). Unfortunately, the nitrogen meter value of head cabbage (Kbs) was exempted due to its unmeasurable thick leaf and pale colour. Seven leafy vegetables, composed as the previous discussion of SPAD value, indicated the presence of nitrogen from

3.9 % ± 0.2 % up to 4.8 % ± 0 .4 % which is fairly higher than those of head cabbage (Kbs), green leaf lettuce (Sel), and sword-leaf lettuce (Sak) whose values were less than 2.4 % ± 0.2 %.

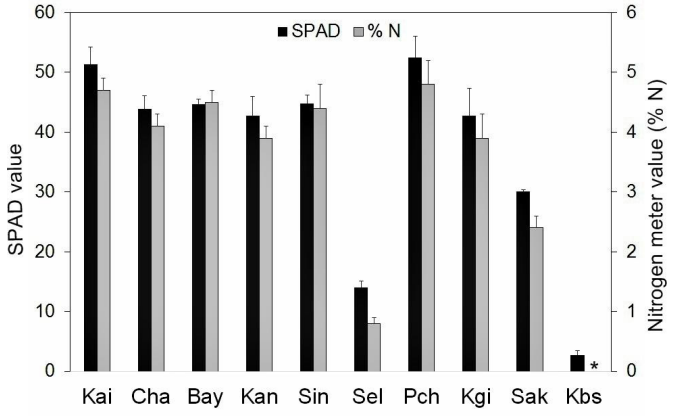

Fig. 1. In vivo analysis of green leafy vegetables by means of SPAD-502 chlorophyll meter and nitrogen meter. Abbreviations were defined in Table 1. *: Head cabbage (Kbs) was unmeasurable by the nitrogen meter.

The measurement by means of chlorophyll and nitrogen meter is based on the principle of detecting absorbance of certain wavelengths which were sent through the leaf tissue. Both systems use particular algorithms for internal calculations in order to produce in vivo data. This method is non-destructive, cheaper, and can be accomplished within a short time after sample collection. However, in vitro quantification is often needed for any verification and comparison purpose. In the present study, HPLC was employed to quantify chlorophyll content of each green leafy vegetable after extraction using organic solvent. Fig. 2 shows data of total peak area, obtained from HPLC measurements, which are proportional to the actual amount of chlorophylls present in the leaf. It was consistent with the result of in vivo measurements using chlorophyll and nitrogen meter, three vegetables showed an average of total peak area < 200 000, i. e. head cabbage (Kbs), green leaf lettuce (Sel), and sword-leaf lettuce (Sak). Nevertheless, the other seven vegetables were not in the same order as observed through in vivo measurement, revealing that in vitro detection is somehow needed for actual data verification.

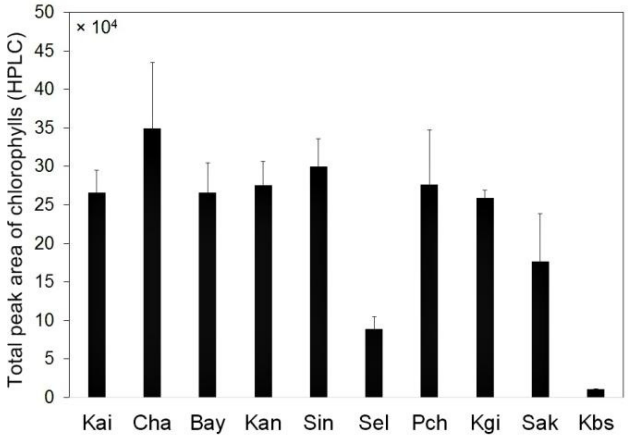

Fig. 2. In vitro analysis of the methanol extract of green leafy vegetables by means of HPLC, expressed as total peak area of chlorophyll *a* and *b* monitored at 400 nm to 700 nm. Abbreviations were defined in Table 1.

In order to find out the distribution of the feasibility of in vivo measurement among ten selected leafy vegetables, compared to their in vitro data, the data plot between total peak area of chlorophylls (HPLC) and SPAD value was generated (Fig. 3a). The best-fit regression was obtained as linear correlation with goodness of fit (R^2) > 0.7, revealing the presence of moderate relation between SPAD value and total peak area of HPLC for chlorophyll *a* and *b*. In green vegetables, chlorophyll degradation will occur along with leaf senescence after harvest[3,10]. Therefore, chlorophyll meter can be employed for rapid detection of vegetable quality and freshness. Several limiting factors are the thickness, morphology of leaf surface, as well as homogeneity of the distribution of chlorophyll in leaves.

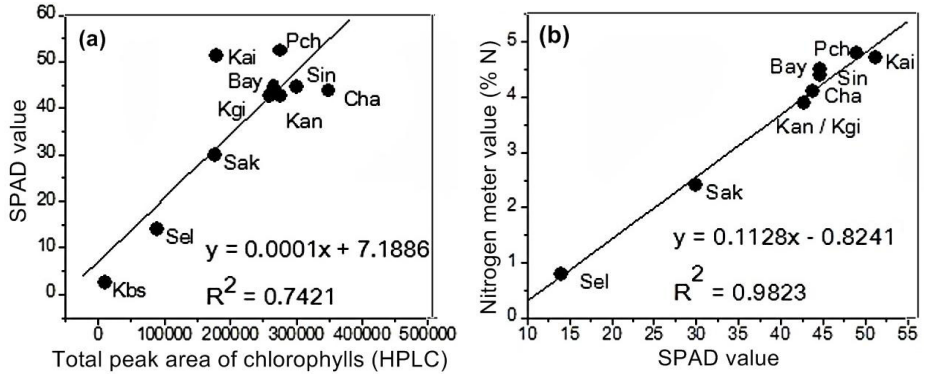

Fig. 3. Data plots of SPAD values versus total peak area of chlorophyll *a* and *b* obtained from HPLC measurement (a), and nitrogen meter versus SPAD values (b). The equation of the linear regression line and its coefficient of determination (R^2-value) is given in the figure. Abbreviations were defined in Table 1.

In addition, appreciable linear fit (R^2 = 0.98) was found in the positive correlation among in vivo measurement (Fig. 3b). This finding is comparable with the result of Johan et al.[6] and Qihua et al.[5], in which a strong linear relationship was observed between SPAD and nitrogen meter values. This is acceptable since the development of nitrogen meter is technically based on the chlorophyll meter (SPAD-502)[6]. However, beside the influence of leaf thickness, the SPAD-502 may be more reliable at very low values, since the lowest SPAD value of Kbs was not recorded by nitrogen meter.

4. Conclusion

Investigation on chlorophyll content of ten species green leafy vegetables which are common in daily diet of local community in Malang, East Java, Indonesia, was carried out. Overall, in vivo (chlorophyll meter and nitrogen meter) and in vitro (HPLC) measurements could provide similar preference of vegetables having significantly high content of chlorophyll than the lower ones. The positive relation between in vivo SPAD values and total peak area of chlorophyll *a* and *b* which was measured in vitro by means of HPLC can be explained as 74 % of the variation in a linear relationship. Furthermore, the SPAD and nitrogen values among all samples showed strong relationship of 98 % based on the similarity of working principle of the devices. The appropriate in vivo detection of photosynthetic pigments on green leafy vegetables is worthwhile for the prediction of freshness and nutritional quality. Several limiting factors, such as leaf thickness, size, and non-uniform distribution of chlorophyll, can be overcome by the development of calibration curve of standard for each species as well as the improvement of measuring capacity of the instrumentation.

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References

1. Qibing W, Jianjun C, Robert HS, Yuncong L. Correlation of visual quality grading and SPAD reading of green-leaved foliage plants. *J Plant Nutr* 2005;28(7):1215–1225.
2. Yan JL, Yi PT, Yong GZ, Hui D, Andrew S. Leaf chlorophyll readings as an indicator for spinach yield and nutritional quality with different nitrogen fertilizer applications. *J Plant Nutr* 2006;29(7):1207–1217.
3. Antonio F, Luca I, Rita M, Giovanni S, Franco T. Colour changes of fresh-cut leafy vegetables during storage. *J Food Agric Environ* 2004;2(3&4):40–44.
4. Antonio F, Tommaso M. Chlorophyll *a* fluorescence measurements to evaluate storage time and temperature of *Valeriana* leafy vegetables. *Postharvest Biol Tech* 2007;45:73–80.
5. Qihua L, Weihua H, Paul J. Use of a SPAD-502 meter to measure leaf chlorophyll concentration in *Arabidopsis thaliana*. *Photosynth Res* 2011;107(2):209–214.
6. Johan UF, Johanna GA, Kristin P, Hakan P. Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. *Photosynth Res* 2007;91:37–46.
7. Dilfuza D, John PAL, Christopher M, Paul LGV. Chlorophyll meters for monitoring foliar nitrogen in three tree species from arid Central Asia. *J Arid Environ* 2012; 85:41–45.
8. Fukshansky L, Martinez A, Remisowsky V, et al. Absorption spectra of leaves corrected for scattering and distributional error: a radiative transfer and absorption statistics treatment. *Photochem Photobiol* 1993;57:538–555.
9. Jan N, Jitka P, Jiri R, Martina S. SPAD chlorophyll meter reading can be pronouncedly affected by chloroplast movement. *Photosynth Res* 2010;105(3):265–271.
10. Xuelian Z, Zhaoqi Z, Jin L, et al. Correlation of leaf senescence and gene expression/activities of chlorophyll degradation enzymes in harvested Chinese flowering cabbage (*Brassica rapa* var. parachinensis). *J Plant Physiol* 2011;168(11):2081–2087.