Adaptation of Latex Diagnosis Parameters Determination using Multiplate Reader and Freeze-Drying Conservation to Support Large-Scale Utilization in Rubber Plantations

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Adaptation of Latex Diagnosis Parameters Determination using Multiplate Reader and Freeze-Drying Conservation to Support Large-Scale Utilization in Rubber Plantations

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Abstract

The Latex Diagnosis (LD) is performed through the determination of dry rubber (DRC), sucrose (Suc), inorganic phosphorus (Pi) and thiol (RSH) contents. It is carried out through the colorimetric method using a single cuvette spectrophotometer, which is time-consuming and requires a high volume of chemicals. It also needs to be executed as soon as the samples are collected so that limits the adoption of LD. The adaptation of the protocol to a multiplate reader and suitable conservation aimed to overcome the limitations. The experiments were carried out at the Indonesian Rubber Research Institute, Sembawa, Palembang, and Research Center for Biotechnology, Universitas Gadjah Mada, Yogyakarta. Various combinations of solution volumes, adapted from standard protocol, were tested. The closest slope of the standard curve to control was then applied to 7 randomly selected trees. The result showed that for Suc, the composition of 10 µL samples, 30 µL of 2.5 % TCA and 210 µL anthrone reagent was suitable for determination using a multiplate reader. The combination of 40 µL of sample, 85 µL of 2.5 % TCA and 125 µL mL $FeSO_4$ solution was suitable for Pi determination, while 100 µL samples, 5 µL DNTB and 100 µL Tris is recommended for RSH. The measurement using a multiplate reader resulted in comparable contents (average deviations were 1.83, 2.69 and 3.51 % for Suc, Pi, and RSH, respectively), lower time and chemicals consumptions, and a larger number of sample handling compared to single cuvette method. This study also tested the effect of freeze-drying for sample conservation. The result indicated that freeze-drying was able to maintain the Pi level (deviation 0.93 %). However, it increased Suc (deviation 19.43 %) and decreased ascorbate (AsA) significantly (deviation 43.60 %); therefore, freeze-drying might not be suitable for conserving serum samples in the LD method.

Keywords: *Hevea brasiliensis*, Latex diagnosis, Sucrose, Inorganic phosphorus, Thiols, Ascorbate, Multiplate reader, Freeze-drying

Introduction

Latex Diagnosis (LD) is carried out through the determination of dry rubber (DRC), sucrose (Suc), inorganic phosphorus (Pi) and thiol (RSH) contents [1,2]. The DRC reflects the latex regeneration between 2 tappings, Suc indicates carbohydrate availability, Pi reflects metabolic activity level, and RSH indicates antioxidant capacity and stress level [3]. Those parameters are used to assess the current physiological status of the rubber tree (*Hevea brasiliensis* Muell. Arg.) in relation to latex metabolism and harvesting-inducted stress.

In the scientific realm, LD is involved in various works, including latex harvesting experiments [4-6], clonal typology characterization [7-9] and plant×environment interactions studies [10,11]. This technology is also utilized in breeding works, such as growth and yield-related investigations [12,13], progenies evaluations [14,15] and performance assessment of rubber clones [16,17]. The practical application of LD in rubber plantations is mainly for yield optimization [18], including tapping panel management [19,20] and ethephon stimulation regime [21].

LD technology is widely used in research institutes, universities and rubber companies [18]. The DRC determination is carried out based on the standard released by the American Standard Testing and Material [22]. For other parameters, the protocols were developed by *Centre de Cooperation Internationale en Recherche Agronomique pour le Development* (CIRAD) based on Dische [23]; Taussky and Shorr [24]; McMullen [25] for Suc, Pi and RSH determination, respectively. The protocol is established using a single cuvette spectrophotometer, which requires a high volume of solution (around 3 mL per sample). As the absorbance measurement is carried out 1 by 1, it is time-consuming which may affect the accuracy of the result particularly when dealing with a large number of samples. A simpler and more robust method of LD quantification is needed for industrial-scale adoption. Quantification using a multiplate reader offers lower chemicals and the ability to handle large samples. However, the protocol for LD parameter quantification using a multiplate reader has not been studied.

The LD analysis should be carried out as soon as samples are collected. A study by Adou *et al.* [26] suggested that the analysis less than 4 h after sample collection was recommended for optimum results. This limits the LD adoption to well-established companies and research institutions, which have on-site laboratories, while smallholding farmers have not been familiar with this technology. In the large-scale adoption, the analysis may not be able to be performed on the same day. Furthermore, for scientific purposes, the samples oftentimes need to be transferred inter-island or even internationally. A suitable conservation protocol will be a significant improvement in LD technology.

Freeze-drying is a well-known conservation method adopted in food processing [27,28], pharmaceutical [29,30] and biotechnology [31]. In *H. brasiliensis*, freeze-dried samples were used for various studies such as carbohydrate reserves [32], protein characterization [33], latex serum composition [34] and enzyme activities [35]. However, the effect of freeze-drying on the LD parameters has never been reported.

This study aimed to establish an LD measurement protocol using a multiplate reader and test the effect of freeze-drying for sample preservation. The measurement using a multiplate reader resulted in comparable contents of LD parameters, lower time and chemical consumptions, and a larger number of sample handling compared to the single cuvette method. On the other hand, freeze-drying might not be suitable for latex serum conservation as it exhibited an increase in Suc and a decrease in the antioxidant (ascorbate (AsA)), although it maintained the Pi level. These results will be useful in promoting the LD method on an industrial scale.

Materials and methods

Research site and plant materials

The study was carried out at Sembawa Research Centre, Indonesian Rubber Research Institute, Palembang and Research Center for Biotechnology, Universitas Gadjah Mada, Yogyakarta, Indonesia from May 2021 to October 2023. The study consisted of 2 experiments i.e. adaptation of latex diagnosis parameters using a multiplate reader and the test of freeze-drying for serum preservation.

For LD quantification using a microplate reader, latex samples were collected from 10-year-old trees of IRR 118 rubber clone at the polyclonal trial 2 (PT2) plot in Sembawa Research Centre, Palembang (2°57'27.1332"S, 104°30'33.57"E). The trees were tapped on the first virgin basal panel (B0-1) every 3 days with 2.5 % ethephon stimulation monthly (harvesting system noted S/2 d3 ET2.5 % 12/y). One mL of fresh latex was collected directly after tapping then added to 9 mL of 2.5 % Trichloroacetic Acid (TCA) in 1 mM of Ethylenediaminetetraacetic Acid (EDTA) and kept on ice. Clear serum was obtained through filtration.

For the freeze-drying experiment, latex was collected from a 15-year-old clone PB 260 at the Agro-technology Innovation Centre of Universitas Gadjah Mada, Yogyakarta (7°55'38.8416"S, 110°24'30.4344"E). The trees were tapped on the first virgin basal panel (B0-1) every 2 days without ethephon stimulation (S/2 d3). Three mL of fresh latex, collected directly after tapping, were added by 27 mL of 2.5 % TCA in 1 mM EDTA and kept on ice. Following a 1-hour transportation to the laboratory, the filtration was carried out to obtain clear serum for further analysis.

Adaptation of latex diagnosis measurement using multiplate reader

The experiment aimed to compare the Suc, Pi and RSH measured using a single cuvette Biowave DNA Spectrophotometer (Biochrom Ltd., Cambridge, England) and SPECTROstar Nano spectrometer (BMG Labtech, Ortenberg, Germany) multiplate reader. The strategy was testing the standard curve using various solutions adapted from the standard LD protocol. The selected combination, the closest slope to control, was then tested using latex serum of 7 randomly selected trees.

Sucrose content quantification

The Suc quantification adopted protocol from Dische [23]. For the standard curve in the single cuvette method, 150 µL of sucrose was added by 500 µL of 2.5 % TCA and 3 mL of 5 mM anthrone (diluted in H_2SO_4) (**Table 1**, treatment A). Following 15 min of water bathing at 90 °C, the absorbance was measured at 627 nm wavelength using a Biowave DNA Spectrophotometer (Biochrom Ltd., Cambridge, England). The absorbance measurement took 40 - 60 s per plate.

In this trial, we tested 4 different combinations of sucrose, TCA and anthrone reagent volumes, analyzed with the multiplate reader, in order to identify the closest slope of the standard curve of the single cuvette method. Flat bottom 96 well plates were used with various volumes as shown in **Table 1**, treatments B - E. Prior to water bathing, the plate was covered by transparent plastic. Water bathing was performed at 90 °C for 15 min. The absorbance was measured using a SPECTROstar Nano spectrometer (BMG Labtech, Ortenberg, Germany) at 627 nm wavelength. The measurement required 5 - 10 s per 96 samples. The selected protocol was the combination of the solutions that had the closest slope to the single cuvette method. This combination was tested on latex serum from 7 rubber trees of clone IRR 118 to compare the sucrose content between single cuvette and multiplate reader.

Table 1 Chemicals volume used for standard curve determination of sucrose content trial.

Chemicals	A	B	C	D	E
	Single cuvette (µL)	Multiplate reader (µL)			
Sucrose solution	150	7	10	10	10
TCA	500	29	25	30	40
Anthrone	3,000	214	215	210	200
Total	3,650	250	250	250	250

Inorganic phosphorus content quantification

The protocol developed by Taussky and Shorr [24] the collaborator was used for Pi quantification. For the standard curve in a single cuvette, 0.5 mL of KH_2PO_4 was added by 1 mL 2.5 % TCA (total 1.5 mL) and reacted with 1 mL $FeSO_4$ solution (prepared by mixing 5 g $FeSO_4 \cdot 7H_2O$, 10 mL of 10 % sulpho-molybdenum, 100 mL distilled water) (**Table 2**, treatment A). Following a 5-min incubation at room temperature, the absorbance measurement was performed at 750 nm wavelength and required 40 - 60 s per sample.

For the standard curve using a multiplate reader, 4 combinations of KH_2PO_4, TCA and $FeSO_4$ volumes were tested as shown in **Table 2**, treatments B - E. The absorbance measurement using multiplate reader took 5 - 10 s per plate. Selected treatment was used for comparison with the single cuvette method using a 7-sample serum of clone IRR 118.

Table 2 Chemicals volume used for standard curve determination of inorganic phosphorus content trial.

Chemicals	A	B	C	D	E
	Single cuvette (µL)	Multiplate reader (µL)			
Standard KH_2PO_4	500	25	40	40	30
TCA	1,000	55	80	85	70
$FeSO_4$	1,000	120	80	125	150
Total	2,500	200	200	250	250

Thiols content quantification

The protocol for RSH quantification followed the method developed by McMullen [25]. For the standard curve in a single cuvette, 1.5 mL of reduced glutathione (GSH) solution was added with 75 µL of 10 mM 5,5'-dithiobis (DTNB) and 1.5 mL of 0.5 M Tris buffer. The reaction lies in the ability of thiols to reduce the Elman's reagent, DTNB. The yellow color will be formed in the process and its intensity increase proportionally with the RSH content [36]. After incubation at room temperature for 30 min, the absorbance was measured at 412 nm wavelength. For the multiplate reader treatments, 4-volume combinations were tested (**Table 3**, treatments B - E). Selected treatment was used for comparison with the single cuvette method using a 7-sample serum of clone IRR 118. The absorbance measurement required 40 - 60 s per sample for single cuvette, while for multiplate reader required 5 - 10 s per plate.

Table 3 Chemicals volume used for standard curve determination of thiols content trial.

Chemicals	A	B	C	D	E
	Single cuvette (µL)	Multiplate reader (µL)			
GSH solution	1,500	100	100	100	100
DNTB solution	75	5	10	20	20
Tris buffer	1,500	100	100	80	100
Total	3,075	205	210	200	220

Freeze-drying experiment

The freeze-drying experiment was carried out on Suc, Pi and antioxidant contents. Due to a low RSH content in latex, 0.5 mM on average according to Junaidi *et al.* [18], quantification of antioxidants was performed on ascorbate (AsA) as it has the highest concentration in latex, ranging from 2.56 to 5.11 mM according to Junaidi *et al.* [37]. For Suc and Pi, latex serum was obtained through filtration of 1 mL of fresh latex added by 9 mL of 2.5 % TCA in 1 mM EDTA. One mL of the aliquot was directly quantified for Suc and Pi, while another 5 mL was freeze-dried using an MCGS Model LGJ-10N/A vacuum freeze dryer (Dongguan Huaxian Ltd., Dongguan, China). Following overnight drying, the pellet was diluted 4 times using 2.5 % TCA in 1 mM EDTA.

For AsA quantification, 1 mL of fresh latex was added to 9 mL of 0.2N HCl supplemented with 1 mM EDTA and stored in liquid nitrogen. Clear serum was obtained through centrifugation at 15.000 rpm for 20 min. One mL of the aliquot was directly analyzed for AsA, while another 5 mL was overnight freeze-dried. The AsA determination was performed by reacting 50 µL of HCl with 50 µL of 200 mM pH 7.5 KH_2PO_4 and incubated at ambient temperature for 30 min. Following the 20 µL distilled water addition, the sample was incubated once again at ambient temperature for 5 min then added 40 µL of 10 % TCA, 50 µL of 44 % H_3PO_4, 40 µL of 65 mM 2,2'-dipyridyl and 20 µL of 110 mM $FeCl_3$. The absorbance measurement was performed at 492 nm wavelength after 1 h of incubation in the oven at 40 °C. All the quantification was carried out using the SPECTROstar Nano spectrometer (BMG Labtech, Ortenberg, Germany).

Statistical analysis

The comparisons between single cuvette protocol to multiplate reader and fresh sample to freeze-dried were performed using paired student t-test at $\alpha = 0.05$. The statistical analysis was carried out using the XLSTAT program version 21.4.63353 (Addinsoft Inc., New York, USA).

Results and discussion

The adoption of LD protocol using a multiplate reader aimed at reducing chemicals and time consumption. Various combinations of samples and reagents were tested to select the combination that produced the closest standard curve to the single cuvette method. The selected method was then compared to the single cuvette method using samples of 7 randomly selected trees. The result would improve the LD protocol in terms of time and accuracy, especially when dealing with a large number of samples.

Sucrose content determination using a multiplate reader

The Suc content determination using the standard single cuvette method used 0.15 mL sample or sucrose solution, 0.5 mL 2.5 % TCA and 3 mL anthrone reagent per sample analyzed. In this trial, 4

combinations of sucrose, TCA and anthrone were tested as shown in **Table 1**. The result indicated that the single cuvette method (control) could produce a standard curve with y = 0.2916x and R^2 = 0.999 (**Figure 1(A)**). All combinations tested in the multiplate reader method produced standard curves with R^2 > 0.99, yet in lower slope compared to the control. Treatment D (10 µL sucrose samples, 30 µL of 2.5 % TCA and 210 µL anthrone reagent) showed the closest slope (y = 0.2817x) to multiplate reader with R^2 = 0.9935 (**Figure 1(D)**). The high slope indicated that the coloration occurred optimally and significant color intensity differences between concentrations were detected. The lowest slope was produced in treatment E (10 µL sucrose samples, 40 µL of 2.5 % TCA and 200 µL anthrone reagent) (**Figure 1(E)**).

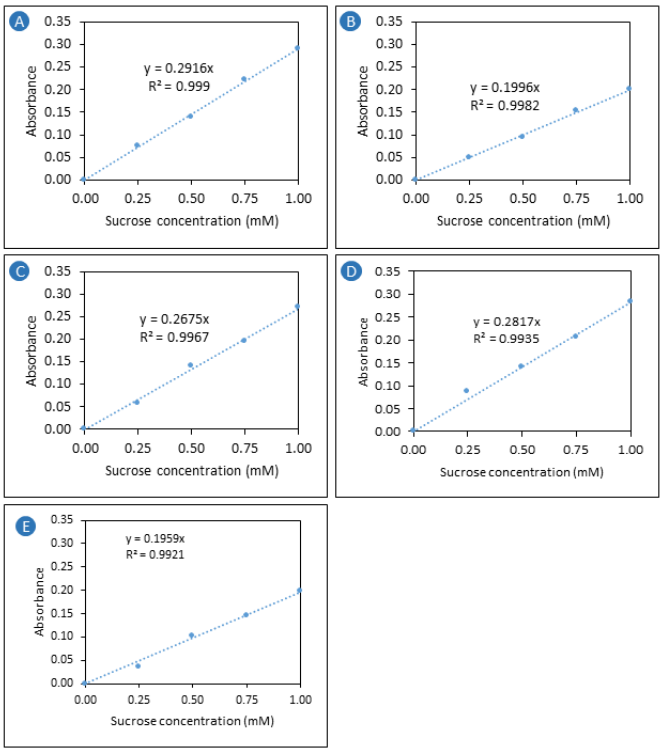

Figure 1 Standard curve of sucrose content determination of the single cuvette spectrophotometer using 0.15 mL sucrose solution, 0.5 mL of 2.5 % TCA and 3 mL anthrone reagent (A); Standard curve of the multiplate reader with 7 µL sucrose solution, 29 µL of 2.5 % TCA and 214 µL anthrone reagent (B); 10 µL sucrose solution, 25 µL of 2.5 % TCA and 215 µL anthrone reagent (C); 10 µL sucrose solution, 30 µL of 2.5 % TCA and 210 µL anthrone reagent (D); and 10 µL sucrose solution, 40 µL of 2.5 % TCA and 200 µL anthrone reagent (E).

Increasing the volume of the sample from 7 µL (treatment B) to 10 µL (treatments C, D and E) increased the slope as shown in treatments C and D. In treatment E, although used 10 µL sample the slope was low as it used high TCA (200 µL). The dilution effect leads to a decrease in the slope. Increasing the anthrone reagent did not linearly increase the slope. Treatment D, with 210 µL of anthrone reagent had a higher slope than treatment B, which used higher volume (214 µL).

The objective of this experiment was to compare Suc measured with single cuvette and multiplate reader using 7 randomly selected serum samples of IRR 118 rubber clone. For the single cuvette (control), the standard 0.15 mL sucrose solution, 0.5 mL of 2.5 % TCA, and 3 mL anthrone reagent was used, while for the multiplate reader, the best combination from the previous experiment (treatment D, used 10 µL

serums, 30 µL TCA 2.5 % and 210 µL anthrone reagent) was applied. The result showed that the average Suc measured using a multiplate reader was not significantly different from that of the single cuvette (**Figure 2**). The average Suc of the multiplate reader was 7.01 ± 3.35 mM, while in the single cuvette was 7.06 ± 3.64 mM (*p*-value 0.845).

The average of Suc in the multiplate reader was 0.05 mM lower than that of the single cuvette with an average deviation of 1.83 %. In terms of chemical consumption, Suc determination using a multiplate reader required lower quantity of sample, TCA and anthrone than that of the single cuvette method. Suc is an important parameter in LD as it represents the carbohydrate availability providing energy for cellular activities, including antioxidants and rubber particle biosynthesis [3]. According to the experiment, treatment D could be adopted for Suc determination using a multiplate reader.

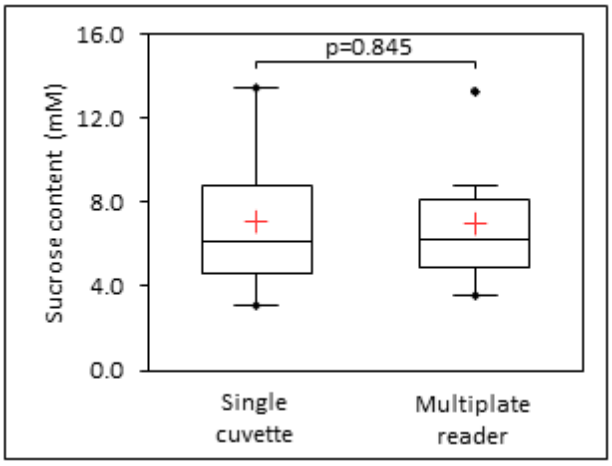

Figure 2 The average sucrose content measured with a single cuvette and multiplate reader. The comparison used 7 randomly selected serum samples of clone IRR 118.

Inorganic phosphorus content determination using a multiplate reader

The Pi determination with the single cuvette used the standard LD protocol of 0.5 mL of standard KH_2PO_4 or latex serum, 1 mL of 2.5 % TCA and 1 mL $FeSO_4$ solution to produce a standard curve with y = 1.1364x and R^2 = 0.9917 (**Figure 3(A)**). Four treatments tested in multiplate reader produced standard curves with $R^2 > 0.99$. The closest slope to control was found in treatment D (40 µL of standard KH_2PO_4, 85 µL of 2.5 % TCA and 125 µL mL $FeSO_4$ solution) with y = 1.0935x and R^2 = 0.9982 (**Figure 3(D)**), while lowest slope was in treatment B with y = 0.721x and R^2 = 0.9997 (**Figure 3(B)**).

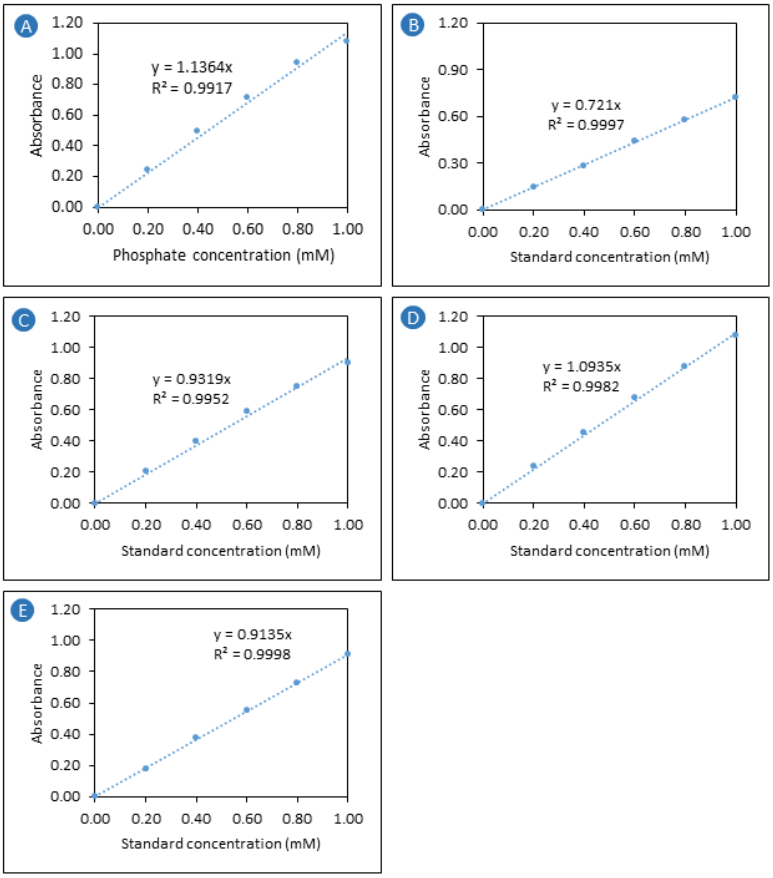

Figure 3 Standard curve of inorganic phosphorus content determination of the single cuvette spectrophotometer using 0.5 mL KH_2PO_4, 1 mL of 2.5 % TCA and 1 mL $FeSO_4$ (A); Standard curve of the multiplate reader with 25 µL KH_2PO_4, 55 µL of 2.5 % TCA and 120 µL $FeSO_4$ (B); 40 µL KH_2PO_4, 80 µL of 2.5 % TCA and 80 µL $FeSO_4$ (C); 40 µL KH_2PO_4, 85 µL of 2.5 % TCA and 125 µL $FeSO_4$ (D); 30 µL KH_2PO_4, 70 µL of 2.5 % TCA and 150 µL $FeSO_4$ (E).

In this experiment, increasing sample volume from 25 µL (treatment B) to 30 µL (treatment E) and 40 µL (treatments C and D) increased the slope. A total volume of 250 µL in each well (treatments D and E) gave a better slope than 200 µL (Treatments B and C). Treatments D and E used a total volume of 250 µL, however, the slope in treatment E was lower than treatment D due to a lower sample volume added (30 µL). Increasing the $FeSO_4$ up to 150 µL did not increase the slope as shown in treatment E. Treatment D showed the best combination and was applied for comparison to the standard single cuvette method.

Determination of Pi was performed on latex serum from 7 trees of clone IRR 118 using the single cuvette and treatment D of multiplate reader. This comparison showed that the average Pi of the multiplate reader (5.17 ± 2.72 mM) was not significantly different from that of the single cuvette (5.56 ± 3.23 mM) (*p*-value = 0.109, **Figure 4**). The average deviation between the multiplate reader and single cuvette was 2.69 %.

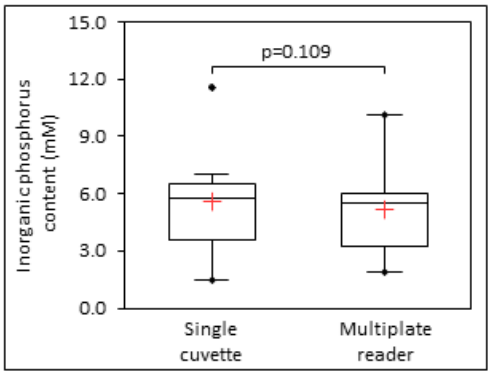

Figure 4 The average inorganic phosphorus content measured with a single cuvette and multiplate reader. The comparison used 7 randomly selected serum samples of clone IRR 118.

In rubber latex, the Pi reflects cellular metabolic activity level [3]. A meta-analysis study by Junaidi *et al.* [18] indicated that Pi and Suc are the main parameters in LD and the most considered for agronomists in settling the harvesting system. In this study, the average Pi using treatment D of multiplate reader was 0.39 mM lower than of single cuvette with an average deviation of 2.69 %. However, the multiplate reader method used less total amount of chemicals than the single cuvette method. The method would decrease LD operational costs, especially on the commercial application scale.

Thiols content determination using multiplate reader

The chemicals used in the standard single cuvette LD protocol for RSH were 1.5 mL standard GSH solution or sample serum, 75 µL of 10 mM DTNB and 1.5 mL tris buffer. The RSH measured using a single cuvette produced a standard curve with $y = 5.3603x$ with $R^2 = 0.9994$ (**Figure 5(A)**). The experiment using a multiplate reader with 4 different combinations was able to produce standard curves with $R^2 > 0.99$ (**Figures 5(B) - 5(E)**). The closest slope to control was gained in treatment B (100 µL GSH samples, 5 µL DNTB and 100 µL Tris buffer) with $y = 3.1591x$ (**Figure 5(B)**), while the lowest slope was in treatment E (100 µL GSH samples, 20 µL DNTB and 100 µL Tris buffer) with $y = 2.9436x$ (**Figure 5(E)**).

According to the experiment, increasing DTNB volume from 5 µL (treatment B) to 10 µL (treatment C) and 20 µL (treatments D and E) did not increase the slope. The 5 µL of DNTB seemed to be the most suitable volume as shown in treatment B. This treatment was tested on 7 samples of clone IRR 118 serum and the result showed that the average RSH of the multiplate reader (0.66 ± 0.21 mM) was not significantly different from that of the single cuvette (0.68 ± 0.18 mM) (p-value = 0.355, **Figure 6**) with the average deviation was 3.51 % to the single cuvette method. Using treatment D of the multiplate reader, the chemicals required only 6.67 % of the sample, 6.67 % of DTNB solution and 6.67 % of tris buffer solution compared to the single cuvette method.

In the summary of the experiment of LD quantification using a multiplate reader, including Suc, Pi and RSH, the measurement using a multiplate reader resulted in comparable results compared to the standard single cuvette LD protocol. The benefits of this method were that it required much less chemicals (6.00 - 12.50 % to single cuvette method). The absorbance measurement using a multiplate reader took only 5 - 10 s for 96 targets, while in single cuvette took 40 - 50 s for each observation on average. Applying a standard single cuvette method, an LD team, usually consisting of 4 people, can manage up to 100 samples per day. Using an adapted multiplate reader, the LD team might be able to handle 100 - 500 samples per day. Thus, the multiplate reader method offered more efficiency in time and better accuracy.

LD quantification based on the colorimetric method, single cuvette or multiplate reader, requires a standard laboratory. It limits the adoption due to high investment of materials and equipment. For future development, a simple real-time measurement, for example using *near-infrared spectroscopy* (NIRS) could be a breakthrough in LD technology application. In *H. brasiliensis*, NIRS has been tested on moisture determination [38], dry rubber content and total solids content determinations [39] and performance prediction of rubber mechanical properties [40]. Nonetheless, the use of NIRS on LD parameter determination has not been reported.

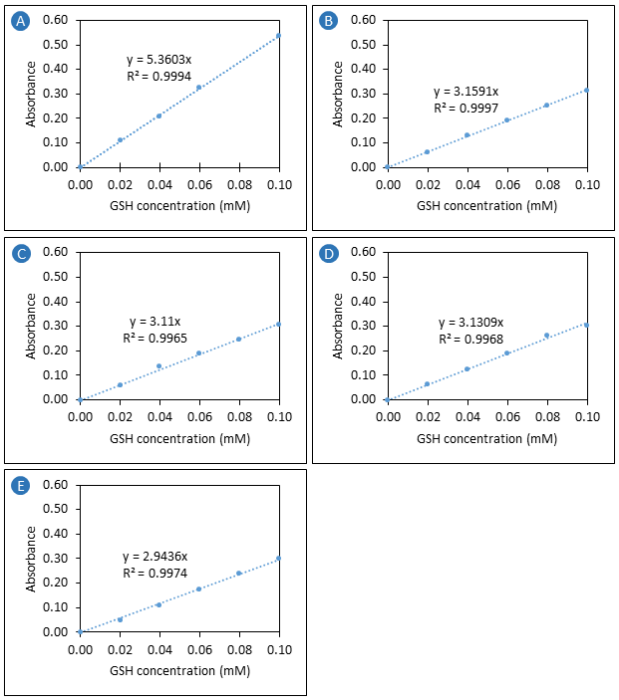

Figure 5 Standard curve of thiol content determination of the single cuvette spectrophotometer using 1.5 mL standard GSH solution or sample serum, 75 µL of 10 mM DTNB and 1.5 mL tris buffer (A). Standard curve of the multiplate reader with 100 µL GSH samples, 5 µL DNTB and 100 µL tris buffer (B), 100 µL GSH samples, 10 µL DNTB and 100 µL tris buffer (C), 100 µL GSH samples, 20 µL DNTB and 80 µL tris buffer (D) and 100 µL GSH samples, 20 µL DNTB and 100 µL Tris buffer (E).

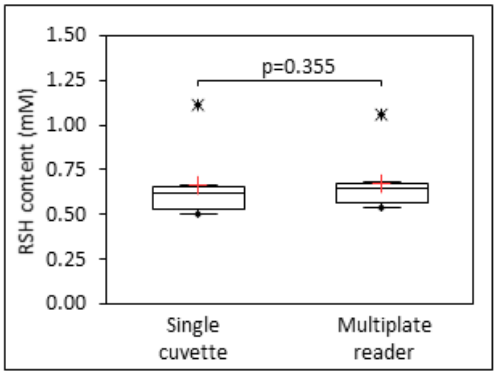

Figure 6 The average thiol content measured with single cuvette and multiplate reader. The comparison used 7 randomly selected serum samples of clone IRR 118 rubber.

Effect of freeze-drying on sucrose, inorganic phosphorus and thiols contents

The freeze-drying effect was studied by comparing the Suc, Pi and AsA of fresh and freeze-dried from the same clone PB 260 samples (**Figure 7**). The result indicated that freeze-dried samples had higher Suc (3.55 ± 0.98 mM) than fresh samples (2.86 ± 1.06 mM) (deviation 19.43 %, *p*-value = 0.047, **Figure 7(A)**). Chakraborty and collaborators reported a similar result of the increment of carbohydrates and total sugar in kiwi fruit powder after freeze-drying [41]. The main principle of the freeze-drying process is sublimation, where water directly transforms from a solid state to a vapor [30]. The absence of water might lead to a higher concentration of non-water constituents, including Suc, though the samples were re-diluted prior to the analysis.

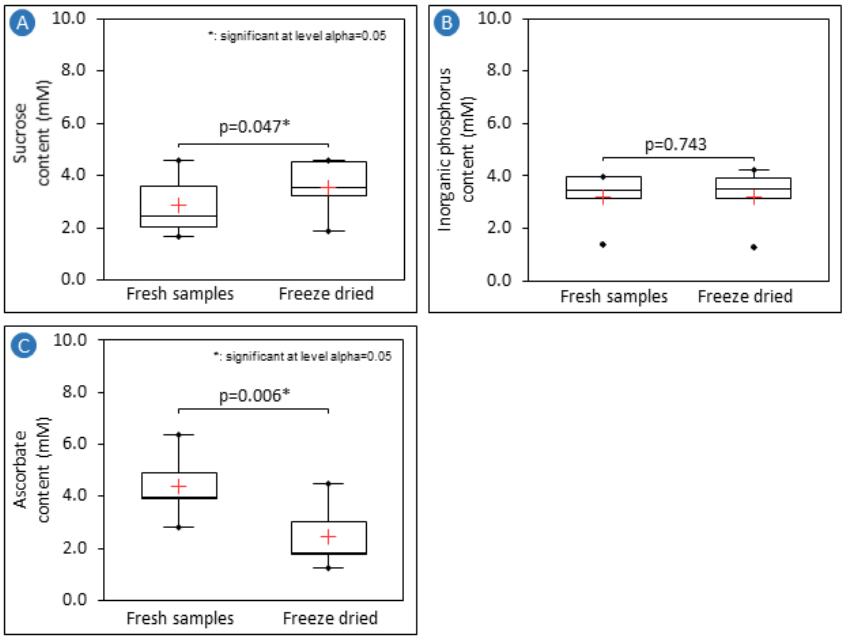

Figure 7 The effect of freeze-drying on sucrose (A), inorganic phosphorus (B) and ascorbate (C) contents. Data were the average of 5 biological replications of clone PB 260.

According to this study, the Pi was not significantly affected by freeze-drying. The average Pi of the freeze-dried sample was 3.18 ± 0.95 mM, while the fresh sample was 3.21 ± 1.04 mM (deviation 0.93 %, *p*-value = 0.743, **Figure 7(B)**). Pi is produced in the process of dissociation of Adenosine Triphosphate (ATP) into Adenosine Diphosphate (ADP) and free energy. The preservation method could cease the metabolic activities, reflected by maintained Pi level after freeze-drying.

In this study, freeze-drying decreased the AsA from 4.38 ± 1.19 mM of fresh samples to 2.47 ± 1.17 mM of freeze-dried samples (deviation 43.60 %, *p*-value = 0.0006, **Figure 7(C)**). The result was in accordance with a study by Jakubczyk and Jaskulska [42], which reported that freeze-drying reduced the activity of antioxidants in vegetable soups. It implied that freeze-drying accelerated the oxidation, producing the Dehydroascorbate (DHA), leading to a decrease in AsA. In the ROS scavenging mechanism, the DHA is reduced back to AsA with the electron source from GSH [43,44]. According to this experiment, freeze-drying might not be suitable for conserving serum samples in the LD method.

Conclusions

This study aimed to establish an LD measurement protocol using a multiplate reader through the adaptation of the standard single cuvette protocol. For Suc, the composition of 10 µL samples, 30 µL of 2.5 % TCA and 210 µL anthrone reagent (total of 250 µL per well) was the most suitable for determination using a multiplate reader. The Pi determination is recommended using 40 µL of sample, 85 µL of 2.5 % TCA and 125 µL $FeSO_4$ solution (total 250 µL per well), while for RSH is 100 µL samples, 5 µL DNTB, and 100 µL Tris (total 205 µL per well). All parameters measured using a multiplate reader with the protocol above showed comparable contents with single cuvette results. In this study, freeze-drying treatment was able to maintain the Pi level, however, this treatment increased Suc and decreased RSH significantly; therefore, it might not be suitable for conserving serum samples in the LD method.

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