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SHORT COMMUNICATION

The CUPRAC-paper microzone plates as a simple and rapid method for total antioxidant capacity determination of plant extract

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Abstract

The current work was aimed to develop the paper microzone plates (PμZP) as a scanometric system for determining the total antioxidant capacity of plant extracts. The PμZP was constructed by immobilizing CUPRAC reagent onto 70-well of patterned paper as a sensing zone for the scanometric detection system. The light blue-sensing zone showed a sensitive response to standard antioxidant (rutin) by forming light yellow color adducts which can be scanned and quantitatively measured by image processing program as a scanometric method. The sensor gave a notable signal at 8 min after rutin addition and presented a linear response in the concentration span of 1–10 mM. The developed method was shown to be reproducible (RSD < 3%) and accurate (98–101% recovery) for determining the TAC of plant extracts (as mM rutin equivalents) and showed strong correlation ($r = 0.9887$) with the standard CUPRAC method, suggesting that it can be applied for the simple and rapid method for TAC determination.

Keywords Antioxidant · CUPRAC · Paper-based sensor · Scanometric · Plant extracts

Introduction

Reactive oxygen species and reactive nitrogen species are naturally generated in our physiology system. As the antioxidant defense systems are insufficient to neutralize these reactive-species completely, the oxidative damage of macromolecules (e.g., DNA, lipids, and proteins) can be posed, and it possibly accounts to the progression of cancer, heart and blood vessel diseases, and the degeneration of the nervous system. Food and beverages rich in antioxidants may be particularly important for giving protection from these diseases as mentioned above [1]. Thus, the development of an analytical method for assessing the antioxidant capacity of the diet with notable antioxidant content has gained importance. The spectrophotometric method employing artificial radicals such as ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) [2], DPPH (1,1′-diphenyl-2-picrylhydrazyl) [3], and DMPD (*N*,*N*-dimethyl-*p*-phenylendiamine) [4] and metal complexes such as ferric-tripyridyltriazine (FRAP method) [5] and cupric-neocuproine (CUPRAC method) [6] are commonly used to calculate the antioxidant activity of synthetic and natural antioxidants and also for determining the total antioxidant capacity (TAC) of foods, beverages, and herbal products.

The CUPRAC method was firstly introduced by Apak et al. [6] as the copper-reducing antioxidant capacity since it quantified the ability of the tested sample to reduce the copper(II) (cupric) ion. It uses the color-forming copper(II)–neocuproine (abbreviated as Cu(II)–Nc) reagent which is readily reduced by antioxidants to form the Cu(I)–Nc chromophore that showed maximum absorbance at 450 nm. The method has been magnificently applied to TAC determination of fruits [7–9], herbal teas [10], plant extracts [11], natural dyes [12], cereals [13], and human serum [14, 15]. Despite its wide use in TAC determination, the CUPRAC method suffers from a large amount of reagent and sample use. To overcome this limitation, the 96-microwell plate was employed by some authors for TAC determination of tea samples [16], stem by-product of grapes [17], grape pomace extracts [18], berry extracts

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[19], and both human urine and serum [20], in which the absorbance reading was done using a microplate reader.

The optical sensor with various signal transduction systems (absorbance, reflectance, luminescence, or fluorescence) has gained more attention recently since they are fit for rapid and low-cost analytical applications. The CUPRAC method was then carried to this technology by immobilizing the CUPRAC reagent onto various solid support matrices such as Nafion [21–23], polymethacrylate [24], or carrageenan [25] into a blue-colored thin film as an optical sensor. TAC determination was typically performed by dipping the sensor in the sample solution with occasional stirring for certain minutes to allow color change. Afterward, the translucent-colored sensor was separated and placed in a cuvette containing distilled water or solvent before absorbance reading. By using this setup, the TAC of mixed antioxidants, tea beverages, fruit juices, plant extracts, and synthetic serum was successfully determined [21, 23–25]. In the case of opaque-colored sensor that may be resulting from a turbid sample solution, the color change of sensor can be measured by using fiberoptic spectrophotometer in reflectance mode. By using this setting, TAC determination of fruit juices, teas, or tissue homogenates can be done without preliminary treatment [22]. However, despite its applicability, the film-based CUPRAC sensor suffered from enormous use of reagent and solvent, as well as time-consuming and tedious fabrication steps. Moreover, an analytical instrument such as UV–Vis or fiberoptic spectrophotometer is demanded to conduct TAC determination.

The paper-based sensor can be visualized as an alternative to the film-based sensor for the analytical device, as the former type is simple, cheap, easy to fabricate and requires a small amount of reagent or sample. The color change of the paper-based sensor as a result of chemical reaction with antioxidants can be photographed or scanned using a smartphone camera or flatbed scanner, as the latter is already known to be scanometric [26, 27]. DPPH was successfully immobilized on the paper to construct an antioxidant sensor [28, 29]. More recently, Folin–Ciocalteu, ABTS, and CUPRAC reagent were separately immobilized on the paper with the consecutive construction of sample, pretreatment, and detection zones which allowed simultaneous detection of total phenolic and antioxidant capacity [30]. In another side, the paper microzone plate (PμZP) has first been developed by Carrilho et al. [31] with 96-microzone design as a substitution for the plastic-based, 96-microwell plate using the photolithography technique. The PμZP was then employed by some authors for the detection of foodborne pathogens [32], tumor markers [33], neuropeptide Y and IgG [34], adrafinil [35], and total phenolic content determination [36–38]. Considering that the CUPRAC method can be done on the 96-microwell plate [16–20], it opened the possibility that the CUPRAC method can be transferred to the PμZP setup.

Based on these facts, we aimed to develop a simple and rapid sensing system for TAC determination in plant extracts by immobilizing CUPRAC reagent onto microzone areas of PμZP as the sensing zone. In practical view, it has a simpler procedure than that of the previous paper-based CUPRAC sensor [30], where the copper chloride with ammonium acetate and the neocuproine was separately immobilized on the detection zone and the pretreatment zone respectively. Intentionally, we applied a scanometric method for processing the digital image data into the chemical-related data as it was used in the aforementioned CUPRAC sensor [30] and in our previous work [26, 27] which made the analysis simpler, faster, and cheaper. To the best of our knowledge, nobody has performed the CUPRAC method in the PμZP and scanometric setup. Here, the fabrication of PμZP and its analytical performance in the scanometric setup are described. The developed method was well applied for the TAC determination of plant extracts as a comparative method for the standard CUPRAC method. The results of both methods in TAC determination were compared, and the relation among the two methods was also evaluated.

Materials and methods

Chemicals

Neocuproine and rutin were bought from Sigma-Aldrich (USA), while CH_3COONH_4 was obtained from Merck (Germany). $CuCl_2.2H_2O$ was obtained from SAP Chemical (Indonesia). Ethanol was obtained from Merck (Germany). Distilled water was obtained from the local market. All chemicals used in this work were of analytical grade.

Plant samples

Guava leaf (red and white fruit variants), turmeric rhizome, and black tea and green tea leaf (in a tea bag) were bought from the traditional market at Jember, Indonesia. All plant materials were individually air dried, ground, and passed through #100 sieves. The plant powder (0.2 g) was ultrasonicated (Elmasonic S180H, Germany) with 10 mL solvent for 10 min. Ethanol was used as an extractive solvent for turmeric rhizome and guava leaf samples, while distilled water was used for black and green tea leaf extraction. The plant extracts were filtered through Whatman filter paper #1 (China) and stored in well-protected tubes in a fridge. For spectrophotometric analysis, all sample extracts were diluted 100 times by using their solvents.

The PμZP fabrication

The paper microzone plates (PμZP) were developed by patterning sheets of paper (Whatman filter paper No. 1, Merck, UK) using screen printing technique with the rubber-based ink. The PμZP design consisted of ten columns and seven rows to make 70 wells that had an internal diameter of 6 mm with an edge to edge distance of 2 mm. Hydrophobic barricades as black circular zones over white-colored background were made by screen printing the rubber ink (Sunrise, Indonesia) on the paper surface. The screen printing process was performed at both sides by superimposing the pattern on one side and on the opposite side of the paper surface to secure the reagent inside the microzone similar to the plastic microwell. Using this method, a number of microzones could be fabricated depending on the desired need. Here, the sensing zone as the antioxidant sensor was fabricated by adsorbing the CUPRAC reagent (3 μL) on the microzones, followed by an air-drying process for 10 min at ambient temperature as depicted in Fig. 1. Afterward, the antioxidant sensor was kept in the well-protected container at 4 °C temperature before TAC determination.

TAC determination using the PμZP-scanometric method

In general, standard antioxidant (i.e., rutin) or sample solution is pipetted (3 μL) and transferred onto the sensing zone. A flatbed scanner (CanoScan, LIDE 110, Japan) is then used to capture the color change of microzone in a color photo mode with 300 dpi resolution. The color intensity of the scanned images (JPEG format) was then quantified with ImageJ program to generate the average value of red (R), green (G), blue (B), or the combined red–green–blue (RGB) color respectively. For analytical purpose, each color value of standard or sample addition was subtracted from that of the blank sensor. The TAC of the sample was computed using the calibration curve of rutin. All of the experimental procedures were performed in triplicate.

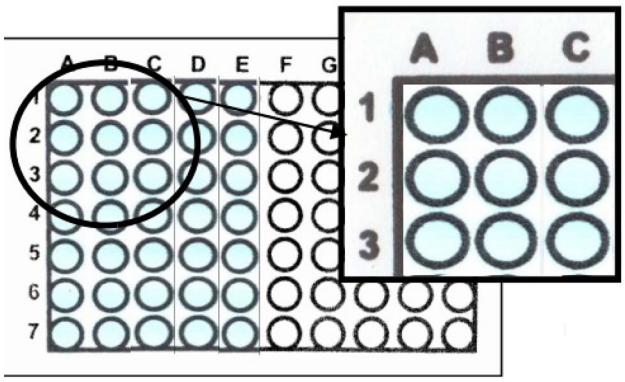

Fig. 1 The PμZP with light blue color sensing zones which functioned as the antioxidant sensor

TAC determination using the spectrophotometric method

The spectrophotometric CUPRAC method was done by following the method described elsewhere [16] with slight modification. Briefly, an equal volume of 0.0075 M neocuproine, 1 M CH_3COONH_4, and 0.01 M $CuCl_2$ solutions were mixed to make the CUPRAC reagent. To the microtube containing 500 μL of the CUPRAC reagent, 500 μL of rutin or sample solution was transferred. After 30 min, the absorbance reading at 450 nm of the mixed solution was done and corrected against the blank (CUPRAC reagent only) using the ELx800 microplate reader (BioTek, USA). The TAC of the sample was computed using the calibration curve of rutin. All of the experimental procedures were performed in triplicate.

Statistical analysis

The TAC value of all samples obtained by the antioxidant sensor and the standard spectrophotometric methods was compared through independent *t* test [39]. To determine the association of the TAC value of both methods, correlation analysis was also employed [40].

Results and discussion

Sensing scheme

The CUPRAC method [6] that quantifies the ability of the antioxidant(s) to reduce cupric ion is the basic sensing mechanism of the current work and the previous CUPRAC-based antioxidant sensors [21–23, 25, 30]. The method uses the blue copper(II)–neocuproine [Cu(II)–Nc] oxidizing-chromogen which can react with antioxidants to yield its reduced yellow colored adduct, the Cu(I)–Nc chelate which absorbs the visible beam maximally at 450 nm wavelength. However, instead of using UV–Vis spectrophotometer [21, 23, 25] or optical spectrometer [22] for quantifying this chelate product as shown in other CUPRAC-based antioxidant sensors, we applied a scanometric method, as well as the paper-based CUPRAC sensor [30] that utilized a flatbed scanner and color image analysis program which is more economical in terms of instrumentation investment and operational-analytical cost.

Optimum color intensity reading

To obtain the optimum analytical signal, different color intensities (R, G, B, and RGB) as sensor responses were compared. The optimum condition was determined by measuring the color intensity change of sensor with the addition of rutin in a serial solution (1–10 mM). As can be seen in Fig. 2, measuring blue color intensity not only gave the highest signal but also exhibited the best correlation coefficient (r) among others. Based on this finding, a blue color reading was applied to all measurements.

Optimization study of sensor reagent

The CUPRAC reagent was employed as a sensor reagent as previously described by some authors [21–24, 30]. For developing the colorimetric antioxidant sensor, the reagent was immobilized onto the surface of solid support films, such as Nafion, polymethacrylate, or carrageenan. While these films have different physicochemical properties from that of paper, the concentration of each component of CUPRAC reagent has to be optimized.

Here, different concentrations (0.8 and 1 M) of $CuCl_2$ and CH_3COONH_4 solutions (8 and 10 M) were mixed with a fixed concentration of a neocuproine solution (0.0075 M) to obtain four formulas of CUPRAC solution as sensor reagents. Each of the sensor reagents was grouped and transferred (3 µL) onto different sensing zones to construct four groups of the antioxidant sensor. For optimization study, a serial concentration of rutin (2–10 mM) was individually applied to each group of the antioxidant sensor. The obtained calibration curves can be seen in Table 1. As the formula *D* gave the highest coefficient of correlation (R) and slope, this formula was selected as the sensor reagent.

Response time

For determining response time, a rutin solution (2 mM) was employed. The sensor response expressed as average blue color intensity (Δ mean B) was monitored every 2 min until a stable blue color intensity value was achieved. The result showed that after 18 min, the sensor response was stabilized, as depicted in Fig. 3. Hence, this response time was applied to the following procedures.

Dynamic range and detection limit

The sensor response of the sensing zone in the PµZP as an antioxidant sensor, at different rutin concentrations and under the optimal experimental condition is shown in Fig. 4. The blue sensing zone gradually changed into yellow color (Fig. 4) just after the application of the rutin solution. Although the color change of the sensing zone can be easily seen by the naked eye, for analytical purpose the color intensity of the

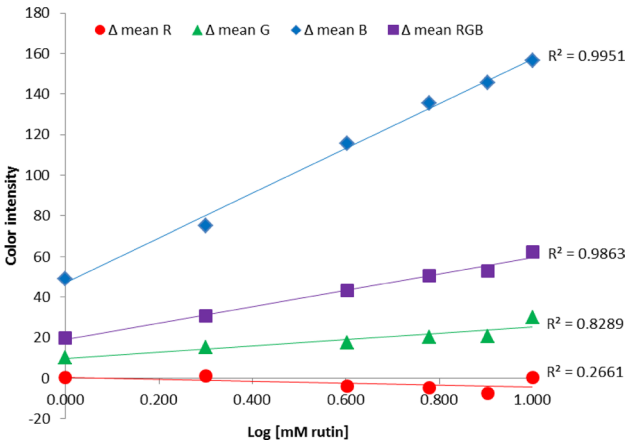

Fig. 2 The observed sensor responses with various color reading towards the addition of rutin (1–10 mM) in 10 min

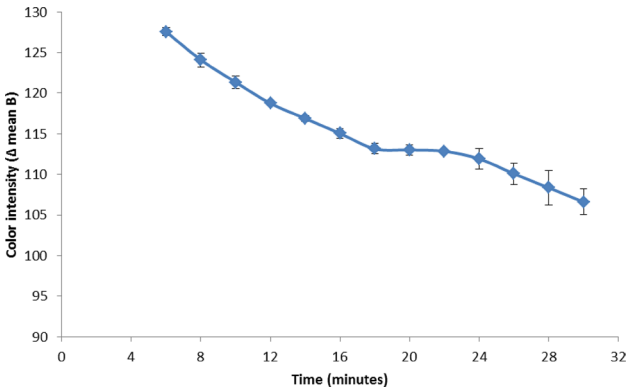

Fig. 3 The observed sensor response towards the addition of 2 mM rutin ($n = 3$) in 30 min

Table 1 The calibration curve of rutin at 2–10 mM concentrations ($n = 3$) within the different compositions of CUPRAC reagent

Formula	Component concentration (M)			R^2	Slope	Intercept
	Neocuproine	$CuCl_2$	CH_3COONH_4			
A	0.0075	0.8	8	0.978	132.85	22.756
B	0.0075	0.8	10	0.991	137.44	27.631
C	0.0075	1.0	8	0.991	140.38	24.591
D	0.0075	1.0	10	0.993	144.99	29.441

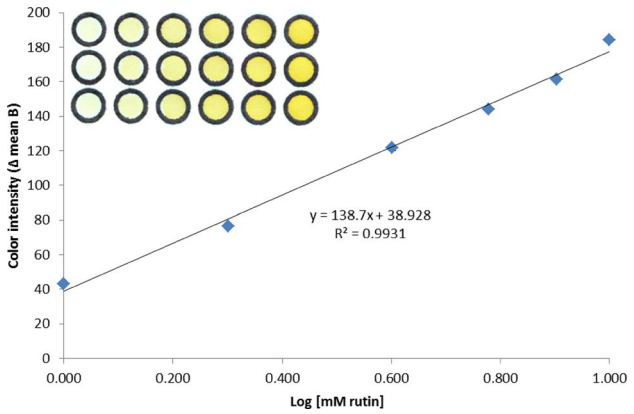

Fig. 4 The calibration curve of rutin constructed by the antioxidant sensor at 18 min ($n=3$)

sensing zone must be quantified by using the scanometric method as previously described elsewhere [26, 27, 41, 42]. The sensor response was found to be linear in the range of 1–10 mM ($r=0.9965$) as depicted in Fig. 4. The sensitivity of sensors obtained from the slope of the calibration curve was computed to be 138.7 Δ mean *B*/mM rutin ($\alpha=0.05$, $n=3$). The detection limit (or LOD) that was obtained from the standard deviation of response times 3.3 was calculated to be 1.119 mM.

Reproducibility

The intermediate precision study was done to assess the performance of the antioxidant sensor in TAC determination by following the method suggested by Yuwono and Indrayanto [43]. Here, TAC of turmeric extract was monitored for 3 days using the antioxidant sensor. As can be seen in Table 2, the relative standard deviation (RSD) values of six replicates were lower than 2.7% in each day, indicating the developed method had good precision [43].

Accuracy

To assess the performance of the developed method in TAC determination, a recovery test suggested by Huber [44] was applied. Here, 2% turmeric extract with TAC value of 8.580 mM RE (equal to 52.383 mg of rutin) was individually spiked with 15.715, 23.572, and 31.430 mg of rutin to represent 30, 45, and 60% of the initial TAC value and analyzed using the antioxidant sensor. As seen in Table 3, the recovery of rutin was found in the range of 98–101%, indicating that the developed method is accurate and shows great potential for determining TAC in plant extracts [44].

Stability

The stability of the antioxidant sensor was evaluated by storing the sensor in various storage conditions. In this regard, the sensor was individually kept in a well-covered container at room (25 °C) and cool (4 °C) temperatures. Afterward, the sensor response within 4 mM rutin addition was monitored every day, until a 15% decrease of the original sensor response was detected. At room temperature, the sensor response was found to decline more than 15% after 90 min, while a similar decrease was observed in 5 days when it was stored at a cool temperature. The stability of the developed sensor was lower than that shown by the paper-based CUPRAC sensor [30]. This may be due to the use of the pre-generated CUPRAC reagent in a mixed solution form which was then immobilized on the surface of paper microzones, as opposed to the CUPRAC above sensor [30] where the copper chloride and ammonium acetate were immobilized on the detection zone, while neocuproine was immobilized in the pretreatment zone. In this regard, the CUPRAC reagent was generated after the sample solution was sequentially moved from the sample zone to the pretreatment zone and the detection zone, respectively.

Application of antioxidant sensor in various plant extracts

While it is commonly known that paper can absorb the colored solution, it is logical to prove that the blue color

Table 3 The recovery of rutin in turmeric extract by using the developed method ($n=3$)

Added mass (%)	Theoretical mass (mg)	Obtained mass (mg)	Recovery (%)
30	68.097	68.9 ± 0.4[a]	101.1 ± 0.6[a]
45	75.955	74.8 ± 1.2[a]	98.5 ± 1.5[a]
60	83.812	82.9 ± 0.7[a]	98.9 ± 0.9[a]
		Mean recovery	99.5 ± 1.4[a]

[a]Obtained mass and recovery round up to one digit after decimal point

Table 2 The total antioxidant capacity of turmeric extract ($n=6$) in 3 days obtained by using the antioxidant sensor

Day	Total antioxidant capacity (mM RE)						Mean	RSD
1	8.668	8.776	8.800	8.674	8.725	8.776	8.736	0.646
2	8.357	8.526	8.459	8.525	8.766	8.414	8.508	1.671
3	8.368	8.435	8.469	8.390	8.489	8.816	8.495	1.932

of antioxidant sensors only changes into yellow color due to redox mechanism rather than color dilution or a combination. Thus, various plant extracts were selected based on their color solution, e.g., turmeric (amber), guava leaf (green), green tea (greenish yellow), and black tea (reddish brown). As these plant extracts were already known for their antioxidant capacity [45–48], these extracts can change the blue CUPRAC reagent into its reduced form (yellow color) inside the microwell plate and on the surface of the microzone (PμZP) as well. Hence, the developed antioxidant sensor can be applied for determining the TAC of the colored extracts.

The TAC values of the antioxidant sensor and the standard spectrophotometric method are presented in Table 4. In general, the result obtained by the sensor was similar to that of a spectrophotometer, suggesting that the developed method was comparable with the standard spectrophotometric method in determining the TAC of plant extracts. Moreover, the obtained TAC values of both methods were highly correlated ($r = 0.9887$) as seen in Fig. 5. In view of the method and operating cost, the proposed method was confirmed to be simpler, faster, and cheaper than the traditional CUPRAC (spectrophotometric-based) method, the film-based CUPRAC sensor method with spectrophotometric system by using absorbance [21, 24] or luminescence detection [22], or even with the more recent paper-based CUPRAC sensor method with scanometric system [30]. The developed method is suited for the field trip analysis as a screening method for TAC determination of plant extracts, as well as it can be applied in the remote area where the analytical instrument is not affordable.

Conclusion

The PμZP as the antioxidant sensor within the scanometric method for TAC determination of plant extract was developed based on immobilized CUPRAC reagent onto filter paper. The response time of the sensor was observed in 8 min which is faster than the analysis time of the standard spectrophotometric method, while the TAC values of plant extracts determined by both methods were comparable and well correlated. The consumption of reagent and sample solution was dramatically reduced, as well as the operating cost for conducting the assay. Hence, the developed method can be viewed as an alternative method for determining TAC or as a screening tool in field trip analysis.

Table 4 The comparison results of the TAC of plant extracts obtained by the antioxidant sensor and UV–Vis Spectrophotometer ($n = 3$)

Sample extracts	Sensor	UV–Vis	$t^{a}_{calculated}$
Turmeric rhizome	8.171 ± 0.092	8.654 ± 0.067	0.0018[b]
Guava leaf M	5.014 ± 0.113	5.336 ± 0.077	0.0150[b]
Guava leaf P	5.570 ± 0.243	5.932 ± 0.097	0.0747[c]
Black tea	5.403 ± 0.081	5.545 ± 0.047	0.0585[c]
Green tea	6.732 ± 0.086	6.829 ± 0.041	0.1549[c]

[a]Results were obtained by independent t test, with t value (t_{table}) of 2.776 ($df = 4$ and $\alpha = 0.05$)

[b]Significant results were obtained within two methods

[c]No significant different results were observed within two methods

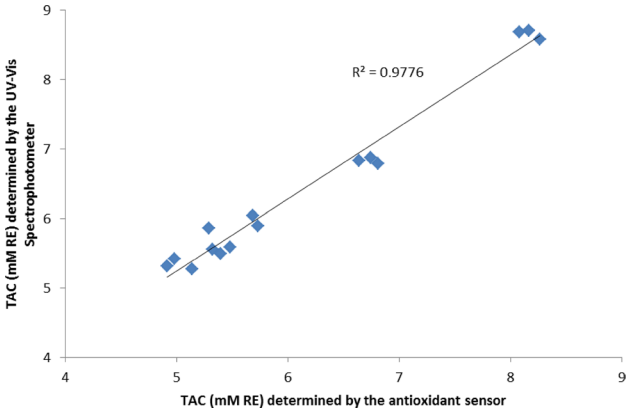

Fig. 5 The correlation of TAC values of plant extracts obtained by the antioxidant sensor with that of UV–Vis spectrophotometer

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements The article does not contain any studies with human or animal subjects.

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