

THE DEVELOPMENT OF AN ALTERNATIVE ALGAL LIPID QUANTIFICATION METHOD BASED ON RESISTANCE OF SCREEN PRINTED CARBON PAPER ELECTRODES

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Abstract

Quantification of algal lipids may require large sample sizes and specialized equipment. Here we present an alternative principle using screen-printed carbon electrodes. Based on a set of experiments using soy bean oil, glyceryl trioleate and crude lipids extracted from *Spirulina (Arthrospira platensis)*, *Ankistrodesmus* sp. and *Chlorella vulgaris*, the normalized resistance of the electrodes changes with concentration of the lipid deposited. The lipid concentration- normalized resistance relationships are of bell patterns with a linear range started from concentration = 0-20 mg/ml and ended around 30-100 mg/ml, depending on lipid types. The sensitivity of the measurement is improved by about an order when fatty acid methyl esters obtained from *Ankistrodesmus* was used instead of the crude extract of the same algae. The technique consumes a dry algal biomass of 0.1-4 mg for an analysis. The amount is much less than that conventionally required for the gravitational analysis (80-100 mg) and smaller than that demanded in the sample preparation for analysis with GC (7-10 mg). The technique has a potential to develop into a cost- effective and less equipment- dependent lipid quantification for microfarms.

Keywords: Algal lipid, biodiesel, lipid quantification, screen-printed carbon electrodes

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Introduction

Among the sustainable energy sources currently known to man, algal lipid derived biodiesels have been recognized as one of the most promising forms (Radakovits *et al.*, 2010; Zhang *et al.*, 2010). The attractive characters of algae as an energy biomass include their high biodiversity (Hu *et al.*, 2008), rapid growth and high productivity (Metting, 1996; Spolaore *et al.*, 2006; Lee, 2011; Moody *et al.*, 2014) (from a few tens to 80% of the drymass (Metting, 1996; Spolaore *et al.*, 2006). It has been predicted that algal biodiesel will replace conventional fossil fuel in the near future (Lee, 2011). Although algal production at a large industrial scale is well established, the trend of algal microfarms is growing (Edwards and Henrikson, 2012; Henrikson and Edwards, 2013). With the development of affordable versions of lab equipment such as home scale algal cultivation systems (Henrikson and Edwards, 2013), and smartphone microscope kits (Kim *et al.*, 2016), a systematic commercial algae microfarm has a great chance to be owned by local farmers. The reduced technological barrier would also allow for microfarm owners to conduct research and development to improve their own algal productions. However, in the case of algal lipid production, one of the major technological hindrances for microfarms is lipid quantification.

In systematic studies and cultivations, quantification of lipid content produced by the algal cultivations determines the optimal harvesting period (Feng *et al.*, 2011). Tracking lipid productivity also ensures the cultivation standard and allows the detection of variations in production (Olofsson *et al.*, 2012). In cultivation optimization, medium formula development and lipid productivity has to be monitored (Woertz *et al.*, 2009; Feng *et al.*, 2011; Yeesang and Cheirsilp, 2011; Lim *et al.*, 2014). Most of the conventional lipid qualification methods rely on the extraction of lipid (Woertz *et al.*, 2009; Feng *et al.*, 2011; Yeesang and Cheirsilp, 2011; Olofsson *et al.*, 2012) (or its derivatives such as fatty acid methyl esters (FAME) (Lepage and Roy, 1984; Yeesang and Cheirsilp, 2011; Lim *et al.*, 2014) from the algal biomass followed by a lipid quantifying method such as weighting (Woertz *et al.*, 2009; Feng *et al.*, 2011; Yeesang and Cheirsilp, 2011) or analysis using gas chromatography (GC) (Lepage and Roy, 1984; Yeesang and Cheirsilp, 2011; Lim *et al.*, 2014). Bligh-Dyer method for algal lipid extraction, using chloroform-methanol-water system (Bligh and Dyer, 1959), and its modification (Folch *et al.*, 1957) are among those widely used. Conversion of algal lipid into FAME has been reported as an alternative methodology to the solvent extraction for

the quantification (Griffiths *et al.*, 2010; Laurens *et al.*, 2012). Traditionally, the obtained extracts can be quantitatively analysed using a balance (Woertz *et al.*, 2009; Feng *et al.*, 2011; Yeesang and Cheirsilp, 2011). Although the weighting is possible in general laboratories, it demands a large volume of biomass (a few 10-100 mg) so that the extract can be handled and weighted accurately. This prevents frequent lipid measurement on small scale cultures such as shaking flasks. GC has been used as a current alternative. It consumes much less sample (i.e. a few μg -mg) in comparison to the gravimetric quantification. However, it requires more technical operation and is costly, especially for microfarms. More recently, a colorimetric method to quantify the algal lipid was reported. It is based on the reaction to produce a copper salt of fatty acid from algal lipid and indirect lipid quantification from fatty acid's copper (Wawrik and Harriman, 2010). However, it entails an intricate sequence of chemical reactions and requires the use of spectrophotometer. The uses of lipophilic fluorescent dye such as Nile Red to stain for and quantify intercellular algal lipids have been demonstrated (Lee *et al.*, 1998; Huang *et al.*, 2009; Lim *et al.*, 2014). Improved protocols to enhance the fluorescent signal and to convert it into lipid quantity have been reported (Chen *et al.*, 2009; Chen *et al.*, 2011). However, the techniques involve handling of expensive fluorescent dye and require access and training to and skill to operate elaborate fluorescence equipment.

In summary, problems with the existing technology to quantify algal lipid, particularly in the case of microfarms are: 1) great dependence of complicated/specialized equipment and/or protocols, 2) requirement of a large amount of sample for the analysis, and 3) cost.

To solve these problems, we introduce a novel approach to quantify algal lipids using screen-printed graphite electrodes on paper. The principle of the measurement is based on the resistive property of the graphite. We speculated that by dropping lipids onto the screen printed graphite, the lipids, which are naturally electrically insulating, would be absorbed and diffused through the hydrophobic contact between graphite particles. Consequently, the graphite particles might be disconnected, resulting in increased electrical resistance, which could be measured by a multimeter. The more the lipid deposited, the higher resistance increased is expected.

In the following section, we will report our results on preliminary tests of the concept by scanning for the detectable change in resistance over

a large range of lipid concentrations dropped on the electrodes. The constructed lipid concentration-changed resistance (R_2/R_1) relationship of different lipid types with more concentration points within a shorter concentration range will also be presented. The characteristics of the curves from different lipids are compared and discussed. The discussion over the future prospect of the technique, in comparison to other methods, and further studies are provided at the end.

Materials and Methods

Lipids and Preparation

Soybean oil (Morakot, Morakot Industries PCL, Thailand) was used in the test of concept experiment. Glycerol trioleate (triolene) ($\geq 97\%$ (TLC), Sigma Aldrich), crude lipids extracted from *Spirulina* (*Arthrospira platensis*), *Ankistrodesmus* sp. and *Chlorella vulgaris* and fatty acid methyl esters (FAME) from *Ankistrodesmus* sp. were used in the in depth experiments. Both Crude lipids and FAME were generously provided by Algal Biotechnology, King Mongkut's University of Technology Thonburi. Crude algal lipids were extracted based on Bligh-Dyer method (Bligh and Dyer, 1959) using chloroform-methanol-water system followed by rotary evaporation to remove the solvent. This type of lipid is among one of the commonly prepared in algal lipid analysis and therefore was used as the main lipid type in our experiment. FAME was also chosen in our experiment as it is one of the less laborious algal lipid derivatives to prepare (Laurens *et al.*, 2012). FAME from *Ankistrodesmus*, one of the well-known lipid producing, was obtained from a treatment of *Ankistrodesmus* lyophilized biomass with methanol-hydrochloric at 80°C for 1 h. The detail protocol can be found elsewhere (Lepage and Roy,

1984). Lipids used in the experiment were diluted to the desired concentration with chloroform.

Electrodes

The electrodes used in this study were screen printed carbon paper type (Model BI1402) bought from Quasense, Thailand. The electrodes were cut into the size of 1.5×5 mm considering the fact that the electrode should be small so it consumes less sample, this electrode size was chosen because it was the smallest that could still be manipulated by hand. The SEM image of the electrode's surface and the electrochemical characterization of the electrode are provided in Figures 1 and 2, respectively.

Lipid Deposition And Resistance Measurement

Prior to the lipid deposition, each electrode was placed on a piece of aluminium foil cut into a size slightly larger than the electrode. The foil also had an embossed rectangular boundary (3×5 mm) in the middle where the electrode sat. It was made by hand, drawing by a pencil on the other size of the foil. This helped protecting the dropped lipid to spread away from the area of electrode. The set up can be seen in Figure 3(a).

In this study, we were interested in the change in the resistance of the electrode due to the lipid deposition. This could be quantified with R_2/R_1 , which is the resistance of the electrode after lipid deposition (R_2) divided by the resistance of the electrode before the lipid deposition (R_1). To record R_1 , the electrode on the aluminium foil was exposed to $4 \mu\text{l}$ of chloroform and let dry. We found that this helped lipid spread uniformly on the latter step. Once dried, R_1 was measured along the length of the electrode using a multimeter (Fluke 175 True RMS Digital Multimeter, Fluke Inc.; and W0452, Wynn's

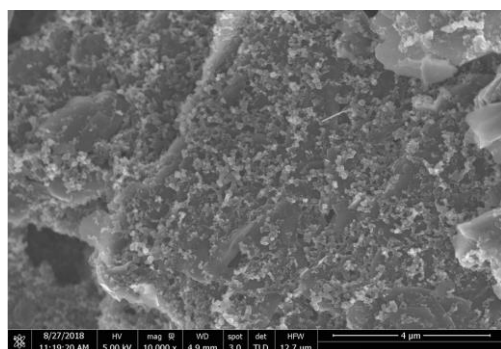


Figure 1. Scanning electron micrograph of the electrode surface. The image is provided by the electrode provider, Quasense Co., Ltd

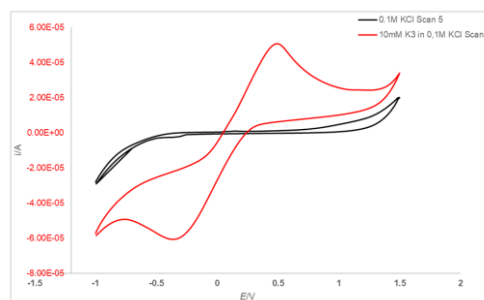


Figure 2. Cyclic Voltammogram of Carbon Screen Printed Electrode on paper with 0.1M KCl (Black line) at scan 5 and 10 mM K_3FeCN_6 in 0.1 M KCl at scan 5 (red line). The current response is $E_{ox} = 0.486 \pm 0.010$ V, $i_{ox} = 4.634 \times 10^{-5} \pm 3.006 \times 10^{-6}$ A, $E_{red} = -0.441 \pm 0.007$ V, $i_{red} = 4.759 \times 10^{-5} \pm 4.091 \times 10^{-6}$ A ($n = 3$). The graph is provided by the electrode provider, Quasense Co., Ltd

tools (Guangzhou) Co., Ltd.). From the measurement, the average R_1 was $0.473 \pm 0.072 \text{ k}\Omega$ ($n = 45$). After that, the lipid deposition was done by dropping 4 μl of lipid solution on the electrode and leaving to dry. This amount of solution sufficiently covered the entire electrode. R_2 was then measured in the same way as R_1 . To ease the measurement, we transferred the electrode to a plastic sheet and placed a pair of rectangular copper electrodes on each side the graphite electrode. To measure the resistance, the multimeter probes were pressed on the copper electrode. This helped avoiding graphite scratching. The 4 mm spacing between the copper electrodes, which was fixed with a pair of plastic spacer, defined a constant measuring distance (Figure 3(b)).

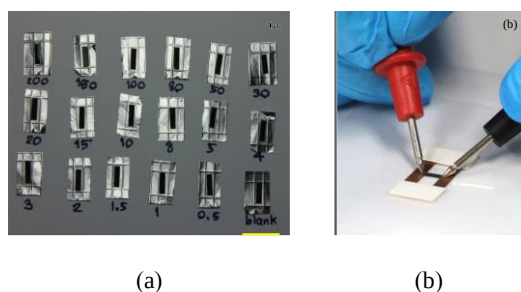


Figure 3. (a) Screen printed carbon electrodes were cut and placed onto pieces of aluminium foil. The rectangular reservoir in the middle was made by pencil marking on the other side of the foil. This prevent the lipid spilling during the deposition. The scale bar represent a length of $\sim 5 \text{ mm}$. (b) A pair of copper electrodes was put on the screen printed carbon electrode before the resistance measurement. The distance of the copper electrodes were fixed with plastic spacer. The copper electrodes help defining the measuring distance and prevent paper electrode to be scratched

Results and Discussion

To test if lipid deposition can cause a detectable change in the electrode's resistance, soybean oil solution in chloroform of a wide range of concentrations (i.e. 0.01, 0.1, 1, 10, 100, and 1,000 mg/ml) were prepared and dropped onto the electrodes. As can be seen in Figure 4, there was a certain range of concentrations at which R_2/R_1 became clearly detectable. R_2/R_1 started rising somewhere around the range of 1-10 mg/ml and peaked at 100 mg/ml (R_2/R_1). Interestingly, R_2/R_1 dropped after 100 mg/ml.

Once the possible working concentration was identified, in depth experiments were carried out on multiple types of lipids including soybean, glyceryl trioleate (triolene) (used as standard equivalence of algal lipid (Chen *et al.*, 2009; Bertozzini *et al.*, 2011) and crude algal lipid extracted from *Spirulina* (*Arthrospira platensis*), *Ankistrodesmus* sp. and *Chlorella vulgaris*. Similar to soybean, all lipids types used could cause a detectable response, as seen in Figure 5. With more concentration points, the lipid concentration- R_2/R_1 relationship can be seen in detail (Figure 5).

Interestingly, the relationship curves of all the lipid samples were distinct from each other but of bell patterns. These results partly support our assumption that the lipid dropped on the electrode might insulate the contact between graphite particles and therefore reduce the conductivity or, in other words, increased the electrodes' resistance. This

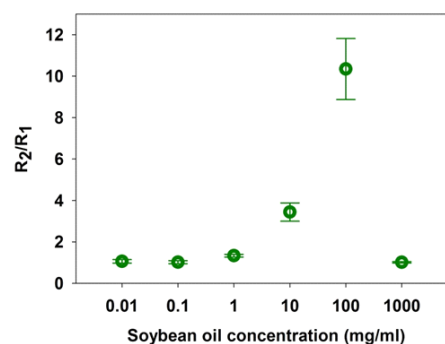


Figure 4. R_2/R_1 of the electrode deposited with soybean oil solution of different concentration. The obvious rise of R_2/R_1 could be seen at 10 and 100 mg/ml. Scatters with error bars represent mean $\pm$ SD ($n = 4-7$)

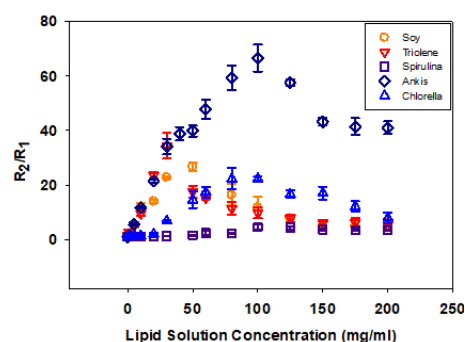


Figure 5. The R_2/R_1 -lipid concentration relationships of different algal lipid samples. All the relationships followed bell curves pattern. Scatters with error bars represent mean $\pm$ SD ($n = 3$)

effect therefore resulted in the beginning of the curves where R_2/R_1 increased linearly with lipid concentration. It cannot be concluded at this point why the relationship became negative after the graphs reached maximum R_2/R_1 . We noticed, however, that the drop in R_2/R_1 coincided with immersion of the electrode surface by lipid solution. We speculate that the drop in R_2/R_1 might relate to graphite mobility on the electrode surface and is a topic for further studies.

Although the lipid concentration- R_2/R_1 relationship was overall non-linear, there was a linear portion that can be used for lipid quantification. Linear regions of the different lipids curves are displayed in Figure 6. In general, the linear ranges started from concentration = 0-20 mg/ml and ended around 30-100 mg/ml making a measuring range of 30-100 mg/ml depending on the lipid types. The R^2 of fitting to a linear function varies from 0.87-0.99. The characteristic of the linear ranges including slope (the sensitivity) and the lipid concentration at which R_2/R_1 reached maxima, were unique to each lipid type as presented in Table 1. We hypothesized that the varied curves' characteristics could be due to the different composition such as pigments and lipid types contained within the samples. In other words, the curves' patterns were a signature of the specific lipid types and could therefore be used to determine type and quality of an unknown lipid sample. The effect of the presence of certain lipid compositions to the graph characteristics could be a matter of a further study.

Beside crudely extracted lipids, we also tested our method on a FAME sample. Interestingly, the FAME obtained from *Ankistrodesmus* gave a greater R_2/R_1 over a broad concentration range in comparison to the crude lipid of the same algae species (Figure 7). Its linear range has a much steeper slope (Figure 8) resulting in a greater sensitivity by roughly an order of magnitude (Table 1). However, the linear range and the R^2 were similar to those of the crude sample of the same algal species. Crude extracts usually contain broad range of molecules from the biomass that are dissolvable in the extracting solvents, some of which could be electrochemically active and interfere with the measurement. This might explain the marked different signalling

patterns between the same algal lipid obtained from different approach. Our finding indicated that the signal is highly dependent on the method of lipid extraction. This is in agreement with previous studies where measured lipids varied with the extractive procedures (Griffiths *et al.*, 2010).

Chemiresistive- based technique has been demonstrated to successfully determining amount of gas and volatile molecules (Steffens *et al.*, 2009; Arena *et al.*, 2010; Mirica *et al.*, 2012). This implies an advantage of the technique that is their ability to sense the target molecules at low amount/concentration. This is of a similar manner to our technique. Considering a 4 μ l sample consumption per test, taking the detectable

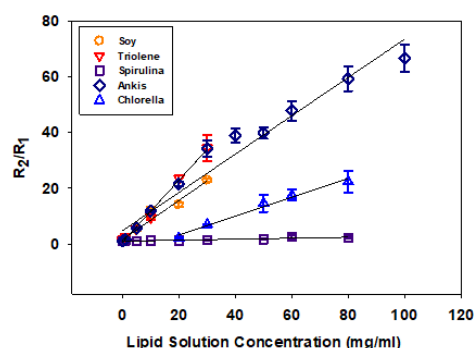


Figure 6. The linear region of R_2/R_1 -lipid concentration curves of different algal lipid. Scatters with error bars represent mean $\pm$ SD (n = 3)

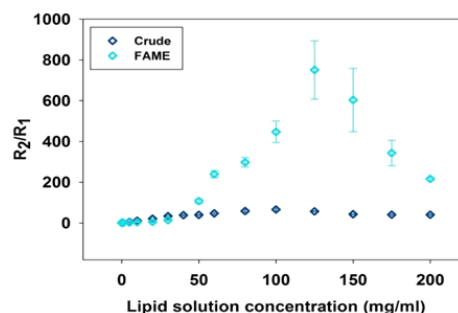


Figure 7. The R_2/R_1 -lipid concentration relationships of crude *Ankistrodesmus* lipid and FAME obtained from *Ankistrodesmus* are compared. Scatters with error bars represent mean $\pm$ SD (n=3)

Table 1. Important characteristic of lipid concentration- R_2/R_1 relationship of different lipid types

Lipids	Approx conc at max R_2/R_1 (mg/ml)	Approx linear range	Sensitivity (R_2 .mg/ R_1 .ml)	R^2
Soybean	50	0-30	0.7025	0.98
Triolene	50	0-30	1.1097	0.99
Spirulina (crude)	100	0-80	0.018	0.87
<i>Chlorella</i> (crude)	80	20-80	0.6871	0.96
<i>Ankistrodesmus</i> (crude)	100	0-100	0.3381	0.98
<i>Ankistrodesmus</i> (demethylated)	125	30-125	7.4466	0.96

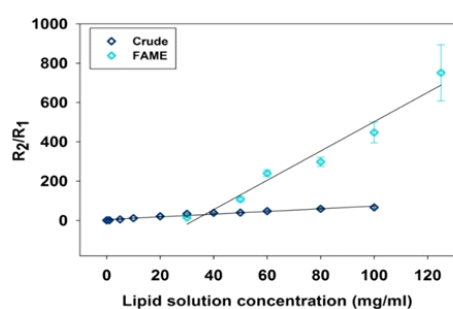


Figure 8. The linear regions of R_2/R_1 -lipid concentration relationships of crude *Ankistrodesmus* lipid and FAME obtained from *Ankistrodesmus* are compared. Scatters with error bars represent mean $\pm$ SD (n = 3)

concentration range to be in an order of 10 mg/ml, the minimal total lipid sample required for the analysis is about 0.04-0.4 mg, equivalent to about 0.1-4 mg algal dry mass, depending on the lipid content in the mass. Hence, this method can clearly reduce the consumption of the algal dry mass used, which is usually around 80-100 mg for gravitational analysis.

In comparison to GC, this sample size is still larger by a few orders of magnitude. It was reported that the LOD of FAME analysis using GC is about 2.5 μ g (Laurens *et al.*, 2012). This means that, in theory, the minimal dry algal mass used for the analysis is in an order of 10 μ g. It has to be noted that the extraction of the lipid is usually conducted on a minimum of around 20 mg dry mass so that accurate dry mass weighing can be done (Laurens *et al.*, 2012). In fact, the GC analysis of FAME was also performed on diluted FAME samples obtained from raw 7-10 mg dry mass (Laurens *et al.*, 2012). This means that the quantity of the sample required by our technique was still smaller than that required by the smallest extraction system for GC analysis. In other words, the sample consumption of our technique was adequately small.

It is obvious that extraction of lipid is a prerequisite to our lipid quantification method. This could be seen as a technical barrier for implementation in microfarms. However, since the technique can be applied to <1 mg dry mass, the extraction system can therefore be greatly scaled down. This is of use especially for extraction by the Bligh-Dyer method. We successfully performed the procedure with a 1.5 ml system in an Eppendorf tube using a dry algal sample of 0.5 mg. The complete process took 2-4 h instead of a whole day and consumed much less reagent. Transesterification of an algal biomass of 10 mg to obtain FAME for the testing usually takes at least 1 h. (Laurens *et al.*, 2012) and is not a problem in terms of duration.

In fact, transesterification is much less complicated in comparison to the Bligh-Dyer method and is a potential sample acquisition method to be used in the future studies.

Other advantages of our technique are in terms of the dependence of technical equipment/protocol and cost of the analysis. Unlike previously reported techniques, which may rely on fluorescent probes (Lee *et al.*, 1998; Chen *et al.*, 2009; Huang *et al.*, 2009; Chen *et al.*, 2011; Lim *et al.*, 2014), spectrophotometry (Lee *et al.*, 1998; Chen *et al.*, 2009; Huang *et al.*, 2009; Wawrik and Harriman, 2010; Chen *et al.*, 2011; Lim *et al.*, 2014) and GC (Lepage and Roy, 1984; Yeesang and Cheirsilp, 2011; Lim *et al.*, 2014), the technique developed here only requires a multimeter and the electrodes, which cost 5-6 pence each. Technical multimeters can be considered elaborate and expensive; budget multimeters are much more general and affordable. In fact, we have performed an experiment using a budget multimeter (W0452, Wynn's tools) and produced a similar pattern of results. This not only allows for the quantification to be conducted using much simpler, inexpensive procedures and equipment, but also reduce the cost per analysis.

The technique is now at the stage of preliminary study. The results, however, reflect its potential for future development into alternative lipid quantification technique not only for general laboratories, but also microfarms. Further studies on the development of graphite screen printed inks specifically for this application could help improve the sensitivity of the measurement.

Conclusions

We reported a set of preliminary studies on the resistive-based technique to quantify algal lipids. The changes in normalized resistance (R_2/R_1) of screen printed carbon electrodes could be observed with a multimeter. The lipid concentration- R_2/R_1 dependencies exhibited bell curve patterns. However, R_2/R_1 increased linearly with increasing lipid concentration at the lower concentration region. Characteristics of the curves were unique to lipid types and lipid extraction methods. The technique was greatly more sensitive to the FAME sample than the crude extract. The technique consumed a low amount of sample (0.04-0.4 mg), which could be provided by a small scale FAME extraction system for GC analysis (about 10 mg dry mass intake). In contrast to other previously reported techniques, our technique promises cost effectiveness, and a lower dependence on equipment and technical protocols. Our preliminary study paves way to the establishment of a new efficient technique for algal

lipid quantification based on resistance which is suitable for general laboratories and microfarms.

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