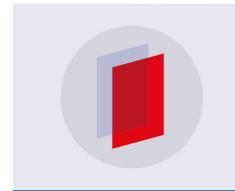
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Rapid reversed-phase high performance liquid chromatography assay of Tert-butylhydroquinone content in food products

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Abstract. The use of antioxidant has been widely applied in food industry to maintain the product's condition to the end consumer. Tert-butylhydroquinone (TBHQ) is a synthetic aromatic organic compound which is often used as antioxidant in food, especially oil product and its derivatives. The use of TBHQ in food products has been limited by the government in most countries, including Indonesia. Therefore, the need for a rapid method to assay the concentration of TBHQ in products is quite high in food industry. In this article, we propose a rapid reversed-phase HPLC method to assay TBHQ content in food products. The isocratic system using 1 % acetic acid and acetonitrile in 3:2 ratio with 1 mL min⁻¹ flow rate produces good separation of TBHQ. Target peak was eluted in 5 min and the whole procedure only need maximum of 18 min to elute all the sample component. The LOD of the method was determined at 0.2 mg L⁻¹, while the lower limit of quantification was 0.5 mg L⁻¹. The accuracy of the method has been proved with acceptable recovery ranging from 95 % to 105 %. **Keywords:** Antioxidant, HPLC, reversed-phase, TBHQ - Tert-butylhydroquinone.

1. Introduction

Antioxidants, both natural and synthetic, are widely used in food products to prevent lipid oxidation during processing, storage, and distribution until they were consumed. Oxidation of lipid results in deterioration of the product quality, especially the flavor and odor. Tert-butyl hydroquinone (TBHQ) is a kind of synthetic phenolic antioxidant (SPA) that is frequently used in frying applications with highly unsaturated vegetable oils. TBHQ is known as the most effective SPA for stabilizing edible oils, in regards of its diphenolic structure and heat stable properties. TBHQ also shows excellent synergism with other antioxidants [1].

As a synthetic antioxidant, the usage of TBHQ in food products is strictly regulated and the both the health risks and benefits have been reported. TBHQ shows a nontypical mode of cell death and proved cytotoxic toward human monocytic leukemia cells, caused apoptosis and significantly promoted DNA damage [1–4]. Along with sodium nitrite, it promoted forestomach carcinogenesis [1]. Otherwise, TBHQ was reported to effectively inhibits cholesterol oxidation and has anticarcinogenic and antimutagenic activities. FDA and USDA permitted the use of TBHQ in food products at less than 0.02 % and 0.01 %, respectively. However, the use of it together with PG is not allowed. Meanwhile, the addition of TBHQ in food products in Japan and Europe is not permitted [1]. The use of TBHQ in

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Indonesia is permitted at concentration less than 180 mg kg for edible oils and 200 mg kg⁻¹ for margarine and similar products [5].

Because of the strict regulations, there is still the need to develop an effective and convenient analysis method for analytically monitoring the use of TBHQ. There are already various methods for determination of TBHQ content in food products. Determination of TBHQ in ethyl esters of fish oil (Icenhour & van Dolah in [6]) and refined vegetable oils by silyl derivatization gas chromatography have been reported (Austin & Wyatt in [7]). Another research reported a simultaneous determination of BHA and TBHQ in biodiesel by batch injection analysis using pulsed-amperometric detection [8]. The most popular method for synthetic antioxidant determination is using HPLC. Reversed-phase HPLC for determination of synthetic phenolic antioxidants including TBHQ in foods have been reported [6, 9]. For more sensitivity, mass spectrometry can be used to detect the eluted TBHQ [10], although MS is not a convenient instrument that always come along with HPLC. The aim of this research is to establish a more rapid and simple method to extract TBHQ from food sample accurately using reversed-phase HPLC with standard UV detector.

2. Materials and methods

2.1. Apparatus

A Waters Alliance 2695 Separations Module, equipped with a quaternary pump with a degasser, an autosampler, a Waters 2487 Dual λ Absorbance Detector, an LC column Waters XBridge C18 (5 μ , 4.6 mm \times 150 mm) was used for TBHQ analysis. A Branson CPX1800 ultrasonic bath, a Hettich EBA 200 centrifuge with the maximum speed of 6 000 rpm (1 rpm = 1/60 Hz), and an AND GR120 analytical balance with 0.000 1 accuracies were also used in this experiment.

2.2. *Materials and reagents*

Fifteen samples including seasoned oil, fry-dried vegetable, fry-dried noodle, palm oil, and palm shortening were purchased from local retail market. Methanol, acetonitrile (gradient grade for chromatography), ethyl acetate (for analysis), and TBHQ (> 98 %) were purchased from Merck. Glacial acetic acid (for HPLC) was purchased from JT.Baker. Water was purified using water distillator system twice to meet the requirement of HPLC grade water (conductivity < 1 μS m $^{-1}$). Nylon filter membrane with 0.45 μm pore size, 25 mm and 47 mm diameter size was purchased from Sigma-Aldrich.

2.3. Preparation of solutions

The mobile phases prepared were 1 % (v/v) acetic acid in water and acetonitrile (ACN). Stock standard solutions of TBHQ (1 000 mg L^{-1}) was prepared in methanol. The stock standard solution was stored at -16 °C and were used for 1 mo. Working standard solutions were prepared fresh daily at concentration ranges 0.20 mg L^{-1} to 50 mg L^{-1} by serial dilution from the stock standard solution. All the mobile phases and standard solutions were filtered through 0.45 μ m filter membrane and degassed before use.

2.4. Sample preparation

Samples were prepared in different methods based on the form. In this experiment, there are three kinds of samples, i.e., solid, semi-solid, and liquid samples.

2.4.1. Solid samples. Solid samples like dried noodle and vegetables were ground with blender and sieved (70 Mesh). The ground samples were accurately weighed to 5 g and then mixed with 50 mL ethyl acetate. The mixture was stirred on a magnetic stirrer for certain minutes (5, 10, 20, and 30) min. After stirring, the mixture was filtered with filter paper and the ethyl acetate was then evaporated with nitrogen flow. The residue was then diluted in 10 mL methanol and mixed vigorously. The mixture

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was centrifuged at 4 000 rpm for 10 min to separate the two phases. Methanol phase of the mixture was obtained, filtered through 0.45 µm filter membrane, degassed, and injected to HPLC.

2.4.2. Semi-solid samples. Samples were accurately weighed to 5 g in an Erlenmeyer. The semi-solid samples, e.g. shortening, were melted at 40 °C prior to mixing with 50 mL ethyl acetate or 10 mL methanol. The ethyl acetate extraction method was the same with the solid sample extraction method. The methanol mixture was vortexed for 5 min. The mixture was then centrifuged at 4 000 rpm for 10 min to separate the two phases. Methanol phase of the mixture was obtained, filtered through 0.45 µm filter membrane, degassed, and injected to HPLC.

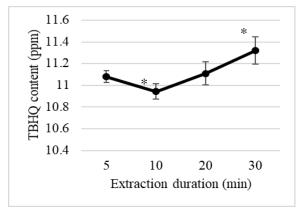
2.5. Liquid samples

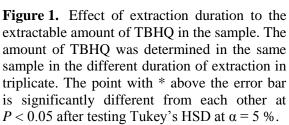
Samples were accurately weighed to 5 g in an Erlenmeyer and then mixed with 50 mL ethyl acetate or 10 mL methanol. The ethyl acetate extraction method was the same as the solid sample extraction method, while the direct methanol extraction method was the same as the one explained in semi-solid samples.

2.6. HPLC analysis

TBHQ were analyzed in standard and sample solutions using isocratic elution. The mobile phase was a mixture of 1 % (v/v) acetic acid in water and acetonitrile. The ratio of the eluent was set to 50:50, 55:45, and 60:40 in order to know the effect of the acetonitrile ratio on the analyte elution. At the start of the analysis, the column was conditioned with $10 \times \text{volume}$ of the mobile phase and waited until the detector signal baseline stable. Precisely, $10 \text{ }\mu\text{L}$ standard or sample solution was injected to the column with a flow rate of 1 mL min⁻¹. The column temperature was set on room temperature (± 20 °C). Detector wavelength absorbance reading was set on 280 nm. The elution runtime was 18 min.

Analyte peak in each sample was identified by comparing the retention time with the one in the standard solution. The data were obtained and processed using Empower 3 software. Statistical analysis of the data obtained was performed using IBM SPSS 20 software with a confidence level of 95 %.





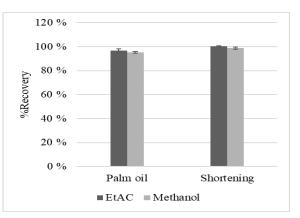


Figure 2. Comparison of two extraction method using ethyl acetate and methanol on the liquid and semi-solid sample. The columns in each sample are not significantly different from each other at P < 0.05.

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3. Results and discussions

3.1. Optimization of the extraction method

The extraction method was developed based on the sample form. The extraction method using ethyl acetate was designed especially for solid sample. The ethyl acetate was used to dissolve the vegetable oil which is usually used in the fry-drying process. To understand the effect of extraction time on the extraction yield, the extraction were conducted in four different time. The data show that 5 min extraction did not give a significant different TBHQ content with 10 min, 20 min, and even 30 min extraction (figure 1). Thus, it can use 5 min, which is the shortest, as the optimum extraction duration.

In this section can be given the hypothesis that the ethyl acetate extraction step was not necessary for semi-solid and liquid samples because in liquid form the samples were ready to be extracted using methanol. Therefore, an extraction procedure without the ethyl acetate step was conducted. Figure 2 shows the comparison of the two extraction method on semi-solid and liquid sample. Apparently, the direct methanol extraction gave a similar recovery with the ethyl acetate method. Thus, we cut off the ethyl acetate step in the extraction procedure for semi-solid and liquid samples. This method allows a more rapid process in the whole TBHQ analysis on semi-solid and liquid samples.

The AOAC official method to determine TBHQ content in oil and butter oil needs extraction procedure using separatory funnel with saturated hexane and acetonitrile as solvent [11]. The whole process is laborious and not efficient. Hao et al. reported an extraction method using only hexane in small sample volume, but with three times extraction [10]. Saad et al. reported another extraction method using MeOH/ACN as solvent with 15 min ultrasonic extraction, 10 min vortex, and an hour settling step [9]. Overall, the proposed sample preparation method in this article is relatively more efficient compared to the other pre-available method.

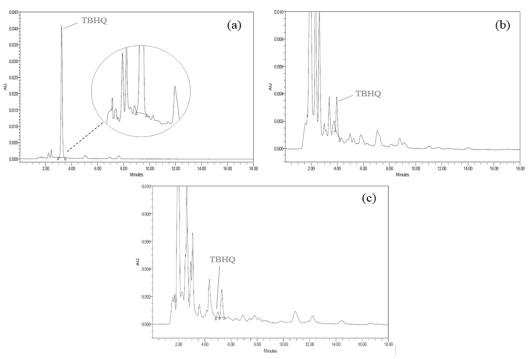


Figure 3. Effect of ACN ratio on the elution of TBHQ. (a) The 50 % ACN ratio gives 3 min retention time of TBHQ in shortening sample, but with bad separation because eluted with the other early eluting components. (b) The 45 % ACN ratio gives 4 min retention time of TBHQ in palm oil sample, but the separation was still not good. (c) The 40 % ACN ratio gives 5 min retention time of TBHQ in palm oil with good separation.

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3.2. Effect of acetonitrile ratio on TBHQ elution in HPLC analysis

Acetonitrile and acetic acid mixture was used as the mobile phase based on the standard AOAC method. The AOAC official method uses gradient system of 1 % acetic acid and MeOH-ACN (1:1, v/v) [11]. Therefore, it is interesting to find more efficient elution system that is suitable for rapid analysis. The proposed method in this research used isocratic elution of 1 % acetic acid-ACN. Gradient elution, especially at high speeds, brings out the limitations of lower quality experimental apparatus, making the results obtained less reproducible in equipment already prone to variation. If the flow rate or mobile phase composition fluctuates, the results will not be reproducible [12]. To achieve the best elution of TBHQ, mobile phases with different ratios of ACN were used to analyze the TBHQ content in several samples.

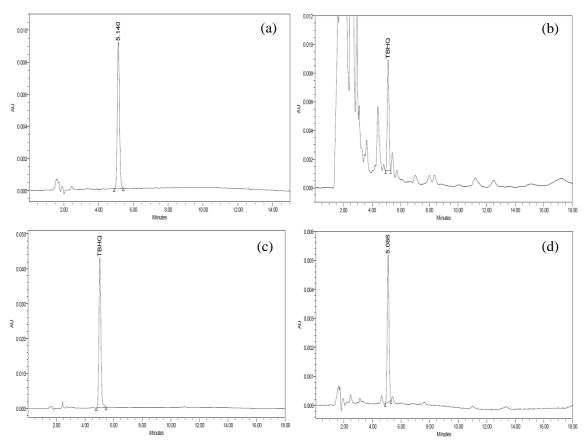


Figure 4. Typical chromatogram of TBHQ separation in optimum condition of (a) standard solution, (b) palm oil extract, (c) shortening extract, and (d) dried noodle extract.

Figure 3a shows the effect of 50 % ACN ratio on the elution of TBHQ in shortening sample. TBHQ was eluted in 3 min as compared to the standard solution retention time (data not shown). This ratio gives a fast elution of TBHQ, but unfortunately it was eluted together with the other early eluting components. This may cause high error in the peak integration and quantification. Furthermore, it lowered the sensitivity of the method because small amount analyte signal may be interfered by the other components' signal resulting in higher detection and quantification limit. The 45 % ACN ratio gives 4 min retention time of TBHQ in the standard solution (data not shown). Elution of TBHQ in palm oil extract showed that the separation was still not good enough to achieve decent integration in sample with small amount of TBHQ (figure 3b). Forty percent ACN seems to be the optimum ratio for the best elution of TBHQ, even in sample with small amount of TBHQ. Figure 3c shows that TBHQ was well detected among the other component, although there was one peak eluted right after TBHQ.

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This peak might not well resolved using the 45 % ACN ratio, as the TBHQ peak was much more shorter in the 40 % ACN ratio compared to the 45 %.

3.3. Method validation

For method validation, the optimum condition of both the extraction procedure and the HPLC condition were used. For the extraction of solid sample, 5 min ethyl acetate extraction was used; for semi-solid and liquid samples, 5 minutes direct methanol extraction was used. The optimum mobile phase ratio for HPLC analysis was 1 % acetic acid-ACN (60:40, v/v). With the optimum method, TBHQ was detected at 5.1 min as seen on the chromatograms of the standard solution and sample extracts (figure 4). The linear calibration curve established by analyzing standard solutions (0.20 mg L⁻¹ to 50.00 mg L⁻¹) was $y = (8.018 \times 10^3)x - (3.514 \times 10^2)$ (R² = 0.9999) (figure 5). The correlation coefficient for TBHQ exceeded 0.999, which indicated that the UV signal response was good enough.

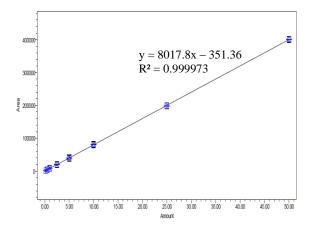


Figure 5. Calibration curve of TBHQ standard solution

The accuracy of the method was checked by determining the recoveries of each sample matrix. Recoveries were determined by spiking the samples with (5, 10, and 15) mg kg⁻¹ TBHQ each in triplicates. The results showed that the recoveries of all sample matrices were between 95.65 % and 104.87 % (table 1), which met the standard of quantitative determination.

The precision of the method was checked with two parameters, i.e. repeatability (intraday precision) and intermediate reproducibility (interday precision). Repeatability was checked by carrying out five replicate analysis on shortening sample containing TBHQ. The relative standard deviation (RSD) of retention time, response area, and response height were 0.63 %, 0.43 %, and 1.10 %, respectively (table 2). Intermediate reproducibility was checked by carrying out duplicate analysis on the same sample containing TBHQ on five different days. The RSD of retention time, response area, and response height were 0.73 %, 0.73 %, and 1.81 %, respectively (table 2).

The use of TBHQ is strictly regulated in most countries and not permitted in some countries, like Japan and European countries. Therefore, the detection limit of the method is an interesting parameter to be known. Analysis of the standard solution could give detection limit of 0.20 mg L⁻¹ (S/N ratio = 15.45) corresponding to 0.40 mg kg⁻¹ in 5 g sample. This detection limit was well below the maximum limit of TBHQ usage in food, which is 200 mg kg⁻¹ [1]. The detection limit of this method is much lower compared to the HPLC-PDA analysis in dry foods (2.0 mg kg⁻¹) [17]; slightly lower than the same HPLC-UV analysis in vegetable oil, butter, and cheese (0.5 mg kg⁻¹) [18]; and slightly higher than LC-ITMS analysis in vegetable oils (0.3 mg kg⁻¹) [10].

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Table 1. The accuracy of the method.

0 1 3/6 / 3	T 1 -	Amount of standard (mg kg ⁻¹)			
Sample Matrix	Level	Spiked	Found	Recovery (%)	
	1	5.000	4.782	95.63	
Palm Oil	2	10.000	9.786	97.86	
	3	15.000	14.348	95.65	
	1	5.000	5.243	104.87	
Dried Noodle	2	10.000	10.057	100.57	
	3	15.000	14.303	95.36	
Shortening	1	5.000	5.172	103.44	
	2	10.000	9.652	96.52	
	3	15.000	14.436	96.24	

Table 2. The precision of the method.

Precision	Parameter	Mean	% RSD
	Retention time	5.099	0.63 %
Intraday	Response area	198 643	0.43 %
	Response Height	22 633	1.10 %
Interday	Retention time	5.091	0.73 %
	Response area	198 582	0.73 %
	Response Height	22 537	1.81 %

3.4. Determination of TBHQ content in real samples

There are various foods which in the production process added with TBHQ to prevent oxidation. Most of the foods are the kind of edible vegetable oil, fats products, and fry-dried foods. One instance of the kind of food containing them all in one package is instant noodle. Instant noodles usually contain fry-dried noodle, fry-dried vegetable, and seasoned oil. In this research, one local seasoned oil, three local fry-dried noodles, two packages of Japan and Europe export quality instant noodle (contain fry-dried noodle, fry-dried vegetable, and seasoned oil), four local palm oil, and one local shortening in the market were analyzed for TBHQ content.

The result of the analyses is shown in table 3. All local products, including seasoned oil 3, fry-dried noodle 3, fry-dried noodle 4, fry-dried noodle 5, all palm oils, and shortening contained TBHQ range between 6.031 mg kg⁻¹ and 168.821 mg kg⁻¹, which is below the maximum limit of TBHQ usage according to Indonesia National Agency of Food and Drug Control (BPOM) (200 mg kg⁻¹ for vegetable oil and 180 mg kg⁻¹ for noodle and shortening) [5]. Products that were labeled as Japan and Europe export quality, i.e. seasoned oil 1, seasoned oil 2, fry-dried noodle 1, and fry-dried noodle 2 contained TBHQ range from not detectable to 1.032 mg kg⁻¹. One sample of fry-dried vegetable was detected of TBHQ residue, but the signal response is below the quantification limit of the method. This finding is interesting yet intriguing because it shows that some of these export goods producers could slipped to ship this non-standard product, although the Japan and EU regulations forbid the use of TBHO in food products. The reason of this error could be a mistake at the production process stage or at the laboratory testing stage. To minimalize production cost, producers sometimes use the same production equipment for both local and export products alternately, which increasing the risks of two products with different specification to be mixed. At the laboratory, the method of analysis used can determine how low the TBHQ content in the products could be detected depends on the sensitivity. Thus, a very low detection limit method is important for analysis in this kind of production environment.

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Table 3. TBHQ content in various commercial food products

No.	Sample Name	TBHQ Conc. (mg L ⁻¹)	n^{b}
1	Seasoned oil 1 ^a	1.032 ± 0.004	3
2	Seasoned oil 2 ^a	n.d. ^c	3
3	Seasoned oil 3	59.42 ± 0.115	5
4	Fry-dried vegetable 1 ^a	n.d.	3
5	Fry-dried vegetable 2 ^a	u.d. ^d	3
6	Fry-dried noodle 1 ^a	n.d.	5
7	Fry-dried noodle 2 ^a	n.d.	5
8	Fry-dried noodle 3	10.946 ± 0.541	5
9	Fry-dried noodle 4	13.08 ± 0.297	3
10	Fry-dried noodle 5	6.031 ± 0.250	3
11	Palm Oil 1	49.858 ± 1.625	5
12	Palm Oil 2	93.128 ± 0.643	5
13	Palm Oil 3	138.223 ± 2.695	5
14	Palm Oil 4	168.821 ± 5.149	5
15	Palm Oil Shortening	125.968 ± 6.210	5

^a Japan or Europe export quality products

4. Conclusions

In conclusion, the proposed method was proved to be capable to be an alternative for determining TBHQ content efficiently in heavily routine environment, such as industry. The method was proved to have good linearity, acceptable accuracy and precision, with decent lower detection limit suitable. With the proposed method, the TBHQ content of various commercial food products were determined. All the local products contain TBHQ at level below the maximum limit in regulations. Most of the Japan and Europe quality export products contain 0 mg L^{-1} to 1 mg/L^{-1} TBHQ.

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b number of replications

c not detected

^d detected, unquantifiable

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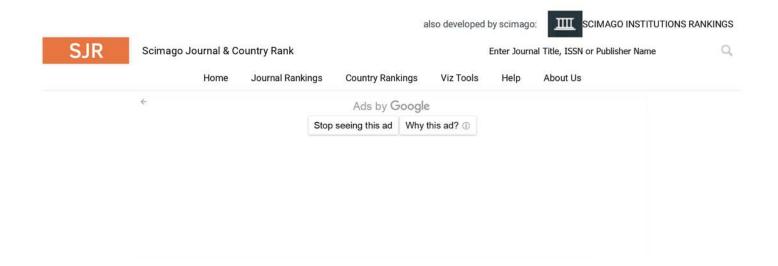
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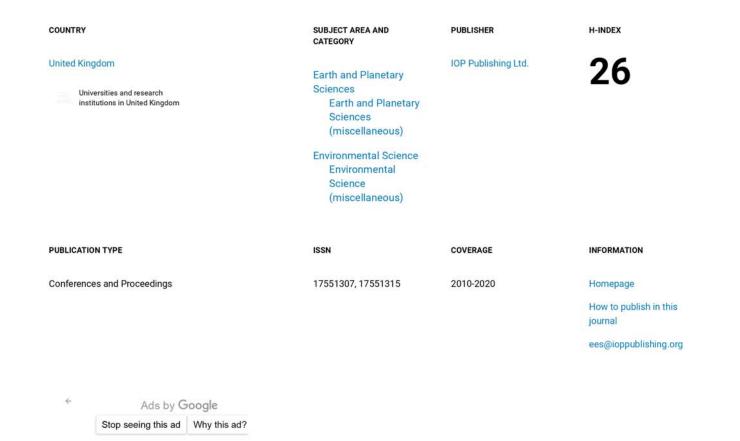
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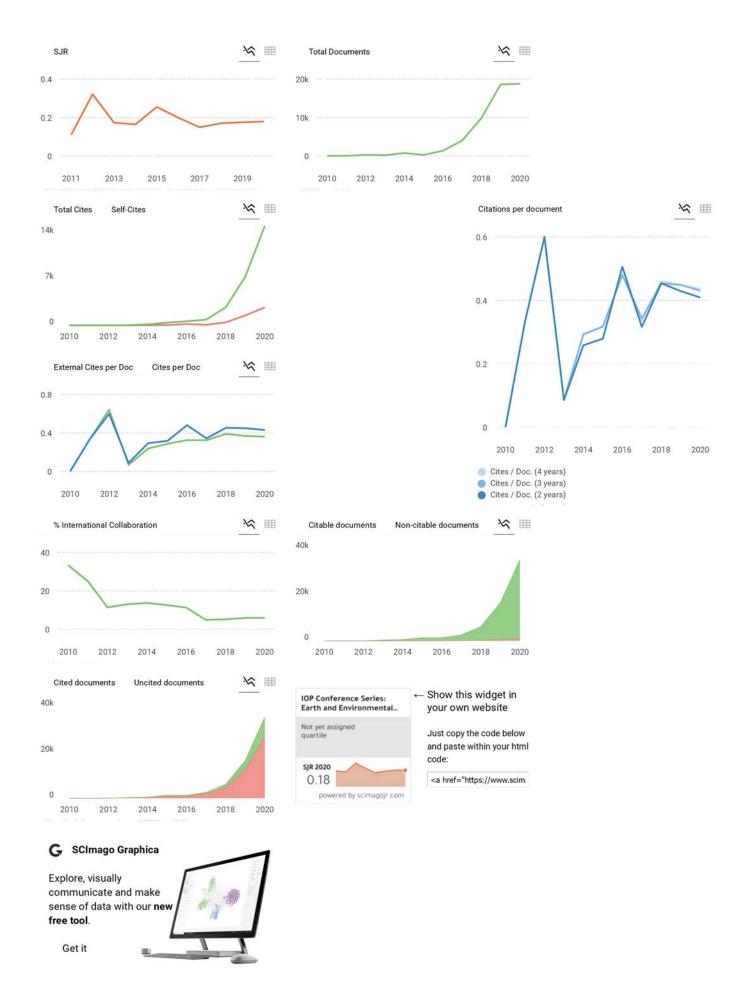
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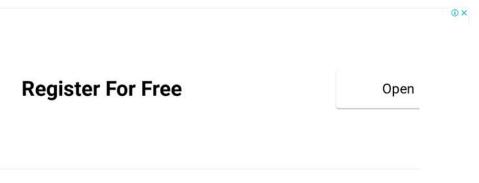
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Melanie Ortiz 2 years ago

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Mursalin 2 years ago

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Please help me to resolve the issue. Thank you for your kindness. I am waiting for good news from you.

Sincerelly,

Mursalin

reply



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syaiful

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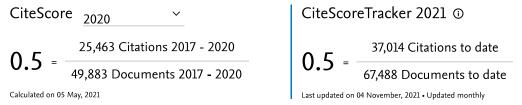
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