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Evaluation of *Apium graveolens* from different geographical origins based on TLC-fingerprint and chemometrics

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Evaluation of *Apium graveolens* from different geographical origins based on TLC-fingerprint and chemometrics

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Abstract. *Apium graveolens* (celery) has various roles both in the food and medicine sectors. It grows very well in the tropical and subtropical areas of Africa and Asia, including Indonesia. This *Apiaceae* member contains a number of phytoconstituents, and geographical origin is known to significantly determine the type and concentration of phytochemicals in plant material. This study was carried out to validate and develop thin layer chromatography (TLC)-based fingerprinting combined with chemometrics, i.e., Principal Component Analysis (PCA) and Cluster Analysis (CA), to evaluate the quality of celery harvested from thirteen different geographical origins in Indonesia. The mobile phase was first optimized with a simplex axial design, resulting in 2-propanol, toluene, and dichloromethane (1:6:1) as the optimum mobile phase for a stable and precise TLC system in the celery sample analysis. When analyzed with chemometrics, the TLC-fingerprints could discriminate celeries from various origins. The PCA score plot of the first two principal components (PCs) and CA clearly distinguished the samples' properties and classified them into four clusters. Samples grouped into one cluster were concluded to have comparable quality, while those in different clusters had different qualities.

Keywords: *Apium graveolens*, celery, cluster analysis, geographical origins, herbal quality, Principal Components Analysis

1. Introduction

Apium graveolens (celery) is a plant that is widely used as a food ingredient in various countries. In Indonesia, it is also used as a component of herbal medicines like *jamu* (a traditional herbal drink), phytopharmaceuticals, and scientifically proven *jamu* to lower high blood pressure. Celery contains various pharmacologically active compounds, especially from the flavonoid group (e.g., apigenin, luteolin, and kaempferol) and the coumarin group (including bergapten and umbelliferone). In addition, volatile compounds like d-limonene, β -pinene, and d-carvone and phenolic acids such as caffeic acid, p-coumaric acid, and chlorogenic acid are also commonly found in this plant [1, 2]. Celery, with these diverse compounds, is known to be a good antioxidant, and this property has been confirmed through various test methods. Furthermore, it also proves to exhibit a protective effect against various metabolic syndromes, such as diabetes mellitus, hyperlipidemia, and hypertension [1, 3].



Apigenin is the main flavonoid in celery and has been shown to have numerous pharmacological activities *in vitro*, *in vivo*, and even in clinical trials [1, 4]. Many have therefore used apigenin levels as a parameter in evaluating the quality of celery [5-9]. Moreover, various pharmacopeias have accepted the use of one or more chemical markers as a parameter of the quality of herbs and their products. Chemical markers can also be used to confirm the correctness of plant species, optimize extraction methods, and even evaluate product stability [10].

Herbal medicine is basically a multicomponent complex whose constituent compounds work synergistically to create pharmacological activities and contribute to its therapeutic safety and efficacy. Therefore, an evaluation that is solely based on one or several certain components cannot thoroughly describe the quality of herbal products [11]. A newer approach called chemical fingerprinting can provide a more comprehensive picture of their characteristics. Several pharmacopeias worldwide have started to accept this strategy, including the Chinese Pharmacopeia [12].

Fingerprinting describes a profile or pattern of characteristics that is chemically representative of herbal composition and usually “displays” as much information as possible. Herbs can be fingerprinted using chromatographic and spectroscopic techniques [12]. Fingerprinting produces multivariate data that require further processing to provide useful particulars of the tested herb. An example includes chemometrics that combines statistics and mathematics to process chemical data into larger and more readily used information [11].

Low and inconsistent quality is one of the challenges that herbal medicines still need to address. Plant sources, geographical environment, and cultivation technology are some of the major factors influencing the quality of herbal medicines [13]. In a previous study, FTIR-fingerprinting followed by chemometrics has been used to analyze celeries collected from ten planting locations [14]. This technique proves effective to classify them and even distinguish three samples of celeries obtained from the market. To complement the celery fingerprinting analysis method, the current research employs a chromatographic technique called thin layer chromatography (TLC). Herbal profile analysis by TLC is a standard procedure that many pharmacopeias still follow, including the Indonesian Herbal Pharmacopeia and the Chinese Pharmacopeia [9, 12].

2. Materials and methods

2.1. Chemicals and plant materials

Apium graveolens was harvested from 13 locations in Indonesia (Table 1), and the samples have been verified by the Center for Information and Development of Traditional Medicine (PIPOT), Faculty of Pharmacy, University of Surabaya, with a certificate of identification No. 1432/D.T/I/2021. Unless stated otherwise, all the chemicals used in this research were obtained from Merck (Darmstadt, Germany). These included TLC silica gel 60G F₂₅₄ plates, methanol, ethanol, toluene, ethyl acetate, formic acid, 2-aminoethyl diphenylborinate, PEG4000, tetrahydrofuran, dioxane, n-hexane, acetic acid, 2-propanol, diethyl ether, dichloromethane, and apigenin (Sigma Aldrich Co., St. Louis, MO, USA).

2.2. Extraction

All parts of the plant that are above the ground were harvested by cutting at the base of the stalk about 2 cm from the ground. Afterward, the leaves were harvested then washed with running water, air-dried, ground into powder, and sifted using a 60-mesh sieve (Figure 1). For each geographical origin, the moisture content of the resulting powder was measured (Table 1). One gram of the powder was extracted with 10 ml of methanol using an ultrasonic bath (42 kHz, 15 minutes) then filtered into a 10ml volumetric flask.

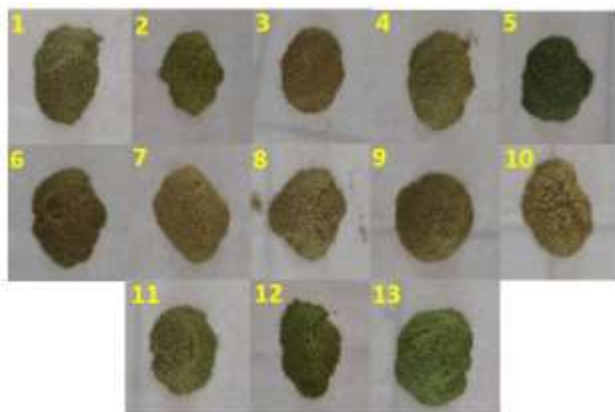


Figure 1. Celery powder from Tawangmangu (1), Materia Medika (2), Batu (3), Pasuruan (4), Bandung (5), Banyumas (6), Mojokerto (7), Ponorogo (8), Bondowoso (9), Karanganyar (10), Lumajang (11), Ngawi (12), and Surabaya (13)

Table 1. Moisture contents of the powdered celeries from different geographical origins in Indonesia.

No.	Location	Latitude, Longitude	Height (m a.s.l)	Moisture content (%)*
1.	Tawangmangu	7°39'54"S, 111°07'45"E	1200	3.03 ± 0.96
2.	Materia Medika	7°52'00"S, 112°31'11"E	896	5.09 ± 1.74
3.	Batu	7°47'36"S, 112°32'17"E	1440	4.21 ± 0.28
4.	Pasuruan	7°53'58"S, 112°49'21"E	1140	3.94 ± 1.07
5.	Bandung	6°49'37"S, 107°35'31"E	1331	3.38 ± 0.18
6.	Banyumas	7°18'16"S, 109°13'26"E	888	3.43 ± 0.39
7.	Mojokerto	7°41'31"S, 112°32'42"E	600	3.52 ± 0.68
8.	Ponorogo	7°52'34"S, 111°35'35"E	355	3.39 ± 0.43
9.	Bondowoso	7°54'51"S, 113°49'21"E	260	4.01 ± 0.53
10.	Karanganyar	7°33'36"S, 110°53'05"E	900	4.83 ± 0.01
11.	Lumajang	7°59'47"S, 113°15'04"E	197	3.89 ± 0.08
12.	Ngawi	7°29'14"S, 111°24'56"E	58	5.12 ± 1.79
13.	Surabaya	7°17'46"S, 112°41'58"E	24	4.96 ± 0.45

*mean ± SD (n = 3)

2.3. Method validation

2.3.1. TLC conditions

The celery extract (8 µl) and apigenin solution (2 µl) were applied to TLC plates. The plate was then inserted into the chamber that had been previously saturated with the mobile phase (according to the *Selection of mobile phase* section). It was eluted to the mark and sprayed with a staining agent (NP-reagent). Afterward, it was observed and documented using a TLC visualizer under visible, 254 nm UV, and 366 nm UV lights.

2.3.2. Selection of mobile phase

The optimum mobile phase was selected using a simplex axial design [15, 16] in two stages. In the first stage, elution was conducted using a single eluent, namely chloroform, tetrahydrofuran, ethanol, dioxane, n-hexane, toluene, ethyl acetate, acetic acid, 2-propanol, diethyl ether, and dichloromethane. Afterward, the second stage mixed two or three of these substances with a predefined ratio as the eluent. The substances selected in this stage were those producing the highest number of compound spots with the best separation in the first stage.

2.3.3. Stability testing based on chromatogram

The stability of the constituent compounds during the elution process was assessed by spotting the extract on the left corner of the plate (10 cm x 10 cm), precisely 15 mm from the bottom and left edges. Afterward, the plate was eluted and dried before being rotated 90° to the left and re-eluted with a new mobile phase.

The compound's stability on the plate and in the extract solution was examined using three differently prepared inputs: (i) the extract was spotted onto the plate and left for three hours before elution, (ii) the extract was prepared and applied immediately before elution, and (iii) the extract was left in a solution for three hours before spotting. The plates were developed and documented using a TLC visualizer.

2.3.4. Precision analysis of the chromatogram

To evaluate the precision, celery powder was weighed three times and extracted in the same way. Then, each extract was applied three times on three different plates. Every time the plate was eluted, the same chamber was used but with a new mobile phase. After elution, the chromatogram was then documented using a TLC visualizer.

2.3.5. TLC-fingerprinting analysis

Celery extracts from 13 different locations were spotted on one TLC plate (20 cm x 10 cm), eluted, and observed under the validated conditions. The chromatogram was then transferred to a VideoScan format to produce the video densitogram and data like Rf values, areas, and peak heights. For every sample, the peak height at each Rf value was then tabulated into a 13 x n data matrix, where 13 represents the number of celery samples and n denotes the number of peaks observed in the video densitogram. The data were then analyzed in Minitab v16.1.0 (Minitab Inc., USA) using Principal Component Analysis (PCA) and Cluster Analysis (CA), which are two chemometric techniques suitable for analyzing multivariate data.

3. Results and discussion

3.1. Optimized mobile phase

Powders of the celeries collected from 13 locations in Indonesia had different organoleptic characteristics (see Figure 1). Sample numbers 5, 11, 12, and 13 were dark green, while the rest were brownish-green. Chemical contents that vary between the plants are believed to be responsible for the color differences, which can be used as the first indicator—i.e., that samples with the same color also have comparable quality. However, this needs to be further confirmed with TLC-fingerprinting.

Sample 1 (from Tawangmangu) was used in the mobile phase optimization and method validation. From eleven single eluents, it was found that 2-propanol, toluene, and dichloromethane produced the best separation. These three mobile phases were thereby mixed with varying ratios using the simplex axial design. The results showed that combining 2-propanol, toluene, and dichloromethane in a ratio of 1.5:6:1.5 created an element with the best separation among the ten prepared ratios. Nevertheless, because the resulting separation was visually less optimal, another mixture with a new ratio was made. Finally, it was found that the ratio of 1:6:1 produced a better separation (Figure 2A).

3.2. Compound's stability

The stability test results of the extract's compounds during the elution process are presented in a chromatogram in Figure 2B. The chromatogram shows that the compound spots lay on a diagonal line after the bidimensional elution. Thus, it can be concluded that the chemical compounds in the celery extract have good stability during elution [17]. Figure 3 shows the stability test results of the compound on the plate (a) and in the extract solution (c). Tracks a and c had the same pattern as track b (comparator). The difference in Rf values of the marker compounds (*) on tracks a, b, and c did not exceed 0.05. In other words, the chemical compounds in the celery extract are stable on the plate and in the extract solution.

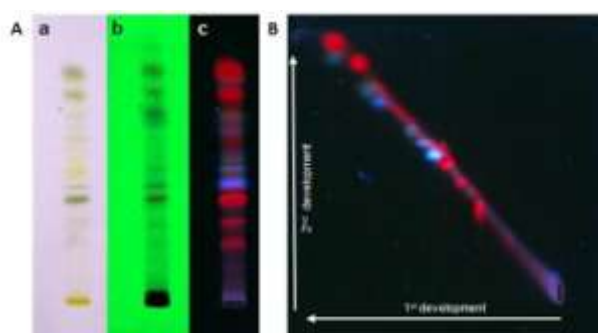


Figure 2.A. TLC plates resulting from the mobile phase optimization for celery extract analysis. **B.** Chromatogram showing the stability of the celery extracts during elution. MP: 2-propanol, toluene, dichloromethane (1:6:1). D: visible light (a), 254 nm UV light (b), 366 nm UV light (c).

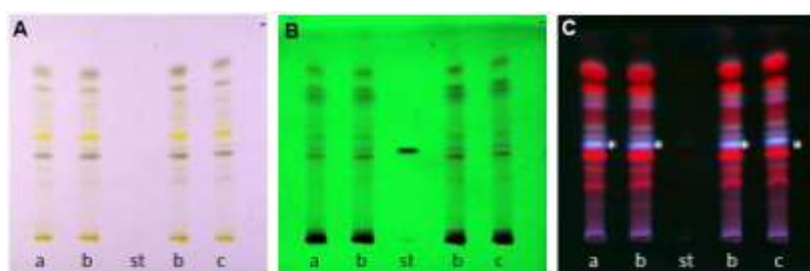


Figure 3. Stability testing of the compound in the extract solution and on the plate with three differently prepared inputs: a. The spotted extract was left on the plate for 3 hours, b: the extract was prepared just before spotting, c: the extract was left in a solution for 3 hours before spotting, st: apigenin. MP: 2-propanol, toluene, dichloromethane (1:6:1). D: visible light (A), 254 nm UV light (B), and 366 nm UV light (C).

3.3. Chromatogram's precision

Figure 4 shows that the TLC method used has good intraday precision, as indicated by the Rf values of the marker compounds (*) that were not higher than 0.02 on the three plates (I, II, III) [17].

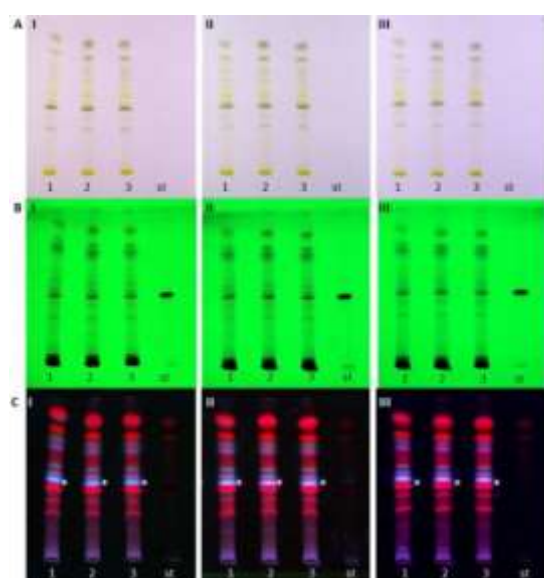


Figure 4. Intraday precision test results. The numbers 1, 2, and 3 mean that the celery extract was spotted three times, st: apigenin, while the Roman numerals I, II, and III show three different plates. MP: 2-propanol, toluene, dichloromethane (1:6:1). D: visible light (A), 254 nm UV light (B), and 366 nm UV light (C). (*) marker compound.

3.4. TLC-fingerprints of celery leaves

Thin layer chromatography (TLC) is an analytical technique that has been commonly used in both qualitative and quantitative analyses of herbs and even for testing biological activities when combined with bioautography. TLC remains a preferred choice for it is fast, simple, and inexpensive. Further,

this method also has great flexibility because it can analyze up to 20 samples simultaneously under the same conditions. With advances in technology, modern high-performance thin layer chromatography (HPTLC) has been successfully developed, thus providing a reliable and robust analytical technique that can meet the demands of Current Good Manufacturing Practices (CGMPs) [18-20].

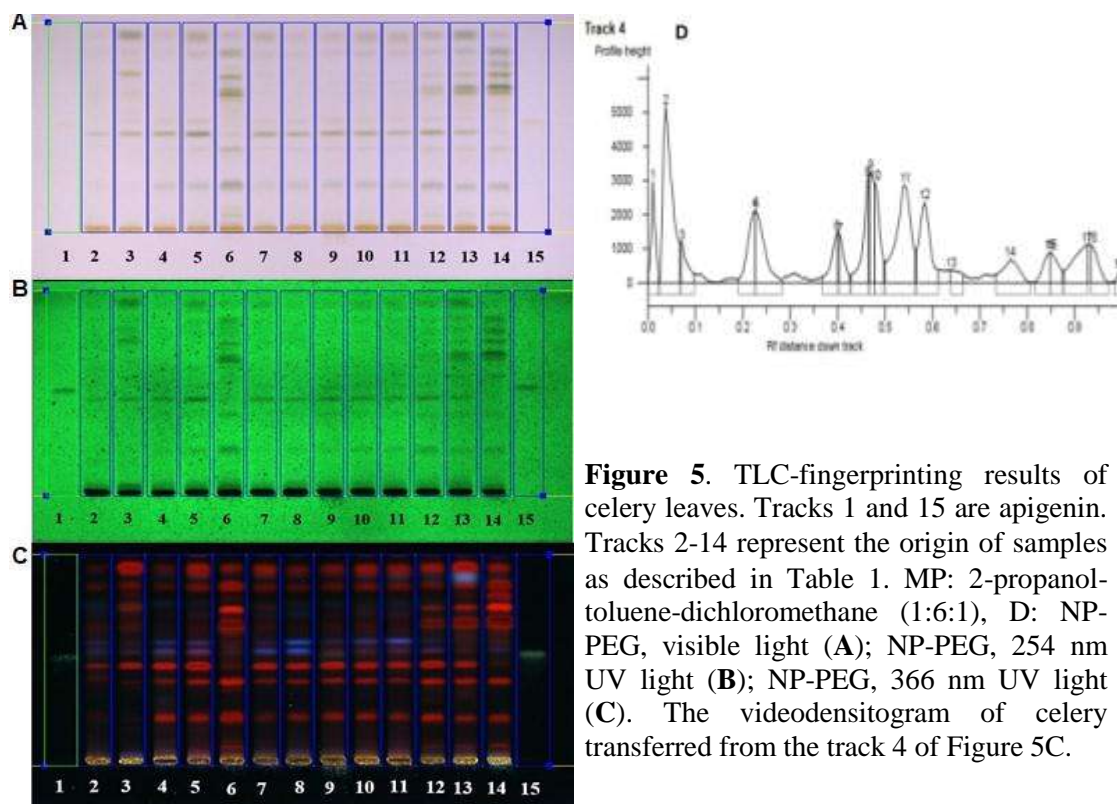


Figure 5. TLC-fingerprinting results of celery leaves. Tracks 1 and 15 are apigenin. Tracks 2-14 represent the origin of samples as described in Table 1. MP: 2-propanol-toluene-dichloromethane (1:6:1), D: NP-PEG, visible light (A); NP-PEG, 254 nm UV light (B); NP-PEG, 366 nm UV light (C). The videodensitogram of celery transferred from the track 4 of Figure 5C.

In this study, the celery leaves (Figure 5) were fingerprinted by spotting the sample extract on a TLC plate (20 cm x 10 cm) using a Linomat 5 TLC, eluting it with 2-propanol-toluene-dichloromethane (1:6:1), and performing derivatization with NP-reagent (1% 2-aminoethyl diphenylborinate in methanol), followed by 5% PEG 4000 in methanol. After 15 minutes, the chromatograms were observed under visible, 254 nm UV, and 366 nm UV lights using a TLC visualizer. Observations with 366 nm UV light (Figure 5C) detected the highest number of spots with the best separation compared with two other lights. Furthermore, in Figure 5, apigenin (tracks 1 and 15) appeared as green fluorescent spots (5C) that reduced fluorescence intensity (5B). However, the samples on other tracks (2-14) did not show any spots with the same color and Rf as apigenin. This is presumably because the apigenin levels in all celery samples are too small to be observed with the TLC system used.

With the TLC visualizer, peaks or patterns on the chromatogram observed under 366 nm UV light (Figure 5D) were converted into a video densitogram (Figure 5D). Afterward, the data presented on it were tabulated to show the Rf value and peak height of each sample (table not shown). These data were obtained from spotting samples three times (triplicates) on three different plates.

3.5. PCA and CA

For each sample, PCA and CA were applied to analyze the height of each detected peak. PCA is a multivariate analysis that can transform original variables that are correlated to each other into new independent ones, creating a smaller dimension that can still explain most of the information contained in the diverse original variables. The scree plot of the PCA (Figure 6A) shows that the total variance was shared among the ten principal components (PCs). PC1 explained 46.5% of the total variance, or the largest proportion compared with the original variables. PC2 contributed 25.6% of the total

variance. It can be concluded that, together, PC1 and PC2 represent 72.1% of the data variability. Accordingly, PCA can reduce data from ten variables (peak heights at ten Rf values) into two new representative ones (up to PC2) because 72.1% of the information can be extracted up to PC2. Figure 6B displays the plot scores of the two principal components (PC1 and PC2) for the thirteen celery leaf samples.

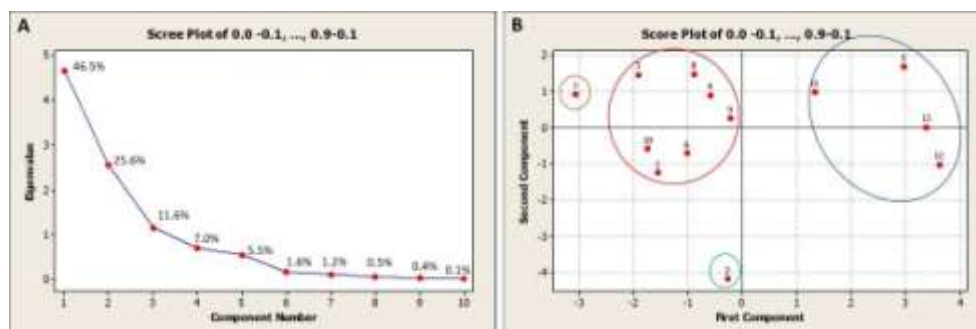


Figure 6. Scree plot of ten PCs analyzed with PCA (A) and score plot of two PCs (PC1 and PC2) from 13 celery leaf samples (B). 1-13 represents the origin of samples as described in Table 1.

The loading plot (Figure 7A) illustrates how strongly each variable is correlated. If two variables are close and form a small angle (e.g., peaks on the chromatogram with Rf values of between 0.1–0.2 and 0.3–0.4), they are positively correlated. On the contrary, if they meet at an angle close to 90° (e.g., peaks on the chromatogram with Rf values between 0.1–0.2 and 0.9–1.0), they are not correlated. Meanwhile, if they are far apart and form a large angle close to 180° (e.g., peaks on the chromatogram with Rf values between 0.5–0.6 and 0.9–1.0), they are likely to be negatively correlated.

After PCA, Cluster Analysis (CA) was applied to group celery leaves from 13 different regions in Indonesia based on their similarity. As seen in Figure 7B, the dendrogram generated by CA shows four clusters of the celery leaf samples. The first cluster consisted of samples from Tawangmangu (1), Batu (3), Ponorogo (8), Pasuruan (4), Banyumas (6), Bondowoso (9), and Karanganyar (10). The second cluster was a sample from Mojokerto (7), the third was from Bandung (5), Lumajang (11), Ngawi (12), and Surabaya (13), while the fourth was from Materia Medika (2).

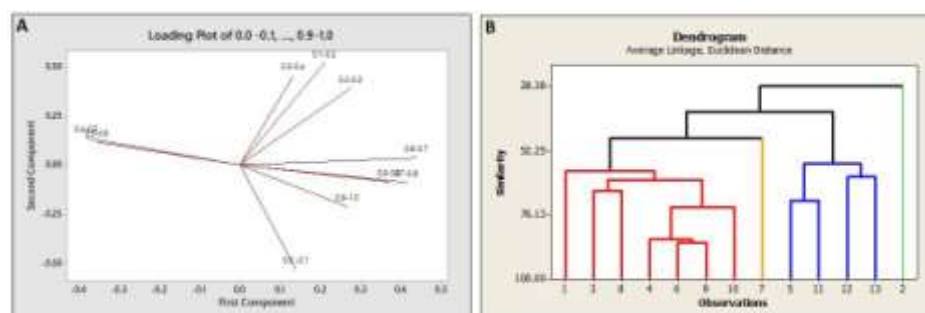


Figure 7. Loading plot of the first two PCs resulting from the PCA of celery leaves from 13 different locations (A). The dendrogram resulting from an average linkage in the CA (B). 1-13 represents the origin of samples as described in Table 1.

Samples grouped into one cluster are likely to have similar chemical content both qualitatively and quantitatively, or in other words, they have comparable quality. On the other hand, different clusters indicate samples with different qualities. The 13 samples of celery leaves observed in this study came from varying heights, from lowlands (Surabaya and Ngawi), midlands (Ponorogo, Bondowoso, Lumajang, and Mojokerto) to highlands (Tawangmangu, Batu, Pasuruan, Banyumas, Karanganyar, Bandung, and Materia Medika). However, the four clusters resulting from the CA are not necessarily related to the altitude of the geographical origin. Other factors such as soil type and cultivation process are believed to affect the clustering. These results are in line with a previous study

that has confirmed the celery leaf clusters produced in the FTIR-fingerprinting are not based on the altitude at which the samples were collected [14].

4. Conclusion


The combination of TLC-fingerprinting and chemometrics (PCA and CA) can distinguish *Apium graveolens* from various locations. *A. graveolens* harvested from 13 regions in Indonesia can be classified into four clusters. Here, the samples grouped into one cluster are concluded to have comparable quality, while different clusters indicate those with different qualities.

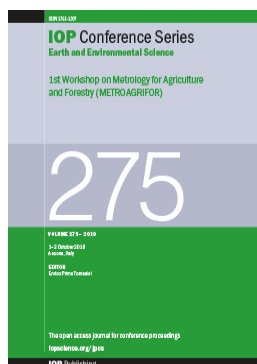
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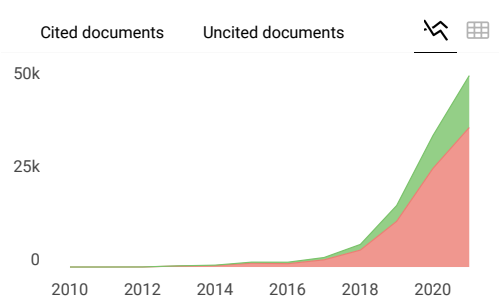
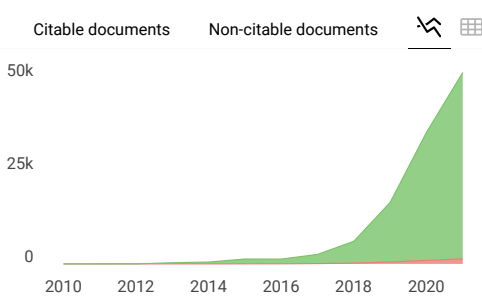
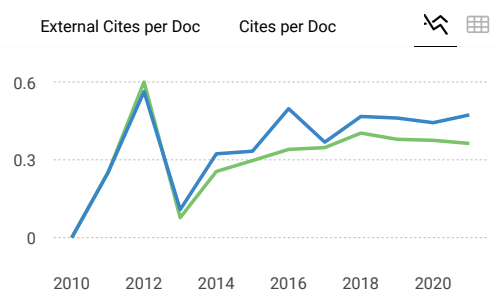
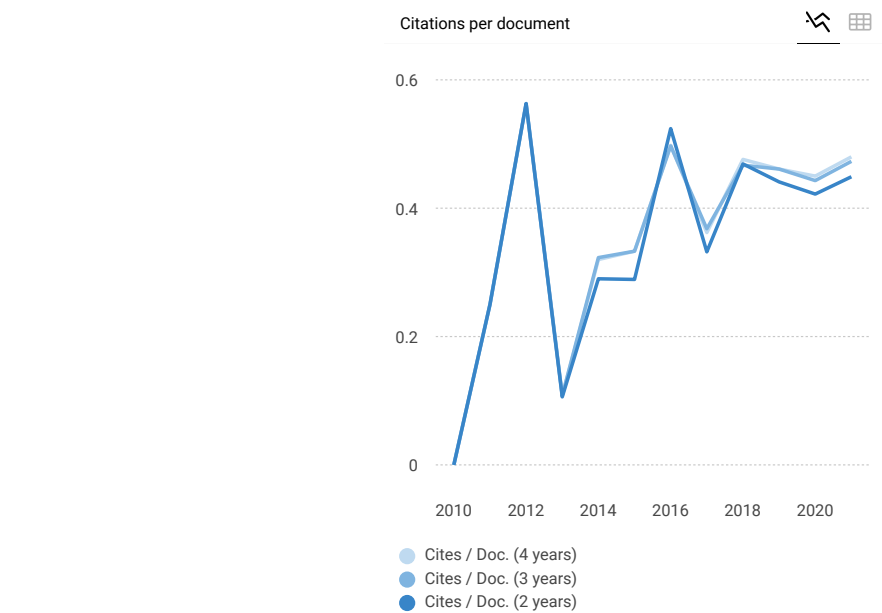
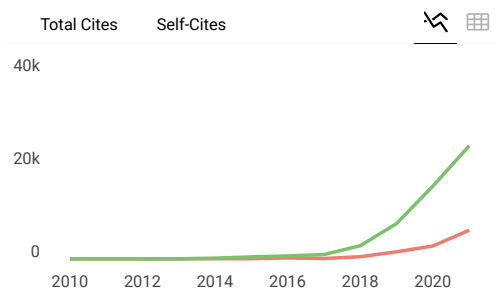
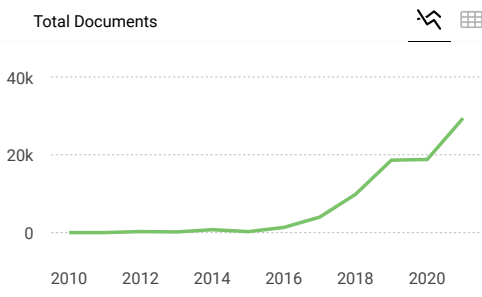
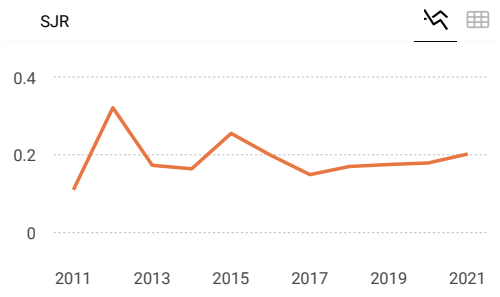
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