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Abstract

Shallots from Brebes, also called Bima Brebes, have a more pungent aroma compared to other varieties. Its high demand results in increased prices in the market, leading to frequent cases of fraud wherein Bima Brebes shallots are replaced with other types of shallots. This study aimed to develop an analytical method using gas chromatography–flame ionization detector (GC-FID) fingerprinting combined with chemometrics to authenticate Bima Brebes shallots. Essential oils were extracted through ultrasonic hydrodistillation, followed by organoleptic, refractive index, GC-FID fingerprinting and chemometric analysis. The yield value of the five studied shallot varieties ranged from 0.02% to 0.08% w/w. Meanwhile, the organoleptic tests and refractive index values showed minimal differences among the five varieties. The GC-FID analysis revealed approximately 149 chromatogram peaks, and chemometric analysis, including principal component analysis, partial least squares-discriminant analysis, and hierarchical cluster analysis, was used to group and differentiate the chromatogram profiles of the five shallot varieties based on their types. Therefore, this method can be used as an alternative analysis technique for authenticating Bima Brebes shallots.

Keywords: authentication, chemometrics, gas chromatography, shallot

Introduction

Shallots (*Allium ascalonicum* L.) are commonly used as a daily food spice and raw material for traditional medicine in Indonesia. The Bima Brebes variety is known for its strong aroma compared to other varieties [1]. Owing to high demand, the market price of shallots has increased. Consequently, some traders sell high-value shallot varieties mixed with cheaper ones, which is detrimental to consumers [2]. Food authentication involves verifying the authenticity or correctness of an ingredient or food product to ensure it complies with labeling [3]. Substituting ingredients that do not meet standards or providing false statements for economic gain is fraud [4].

Although fraud cases involving the Bima Brebes variety, such as replacing it with another type of shallot, do not pose health risks, they can diminish consumer confidence and impact the food industry [5]. This can be observed in the adulteration of saffron powder in the United States, wherein high prices and scarcity led individuals to mix it with turmeric and marigold, which cost less. This causes significant economic losses in the global food industry [6]. Currently, authentication is primarily based on morphological characteristics or plant appearance [7]. However, this method is inaccurate as the color, appearance, and texture of different varieties are often similar [8].

The distinct aroma of the Bima Brebes variety is associated with variations in secondary metabolites, particularly essential oil content. These variations are influenced by genetic differences, geographical origin, and the growing environment, which is a form of plant adaptation. Gas chromatography (GC) analysis can determine differences in the profile of volatile essential oil content [9, 10]. However, because of the complexity of the components in shallot essential oil, a non-targeted approach such as fingerprinting is warranted, as it captures signals from known and unknown compounds [8]. In this study, the flame ionization detector (FID) was suitable for combining with GC because it is a universal detector, making it appropriate for analysis using the fingerprinting approach [11].

However, this method produces chromatogram data with complex patterns, requiring interpretation using chemometric methods to facilitate analysis [12]. Furthermore, in the current study, chemometric analysis involved principal component analysis (PCA), hierarchical cluster analysis (HCA), and partial least square discriminant

analysis (PLS-DA), which are commonly used in food authentication [7]. Morozzi *et al*. [6] demonstrated the successful application of GC-FID fingerprinting combined with chemometrics in saffron authentication. Therefore, GC-FID fingerprinting combined with chemometrics can be utilized to authenticate or differentiate Bima Brebes shallots from other varieties.

Materials and Methods

Materials. Five varieties of shallots were used: Bima Brebes, Biru Lancor, Tajuk, Bauji, and Batu Ijo. The shallots were obtained from certified commercial producers. Moreover, other chemicals were utilized, namely, n-hexane (Bratachem, purity > 99%), anhydrous sodium sulfate (purity > 98%), and distilled water. Additionally, the study used various tools, including GC-FID (Agilent 7890A, Agilent Technologies, USA), ultrasonics (Hielscher Ultrasound Technology, Germany), a set of water distillation tools, a digital refractometer (Atago RX-7000α, USA), a set of glassware, an analytical scale, a blender, a thermometer, a micropipette, OpenChrom software, GCAlignR with R Studio, and MetaboAnalyst 6.0 [13].

Essential oil extraction. Based on the method by Ferdiansyah *et al*. [10] and Ikram *et al*. [14], shallot essential oils was extracted as follows. First, 2 kg of fresh shallot bulbs were weighed, and the shoots and roots were removed. Then, the bulbs were ground and blended with 2 L of distilled water. The mixture was transferred to a container and ultrasonicated for 60 minutes at a temperature of 50 ℃–60 ℃. Further, extraction was performed using the hydrodistillation method with two distillation flasks with a capacity of 2 L, with each distillation flask filled with 1 kg of sample and added with 500 mL of distilled water. Distillation was conducted for 5 hours until shallot essential oil was produced. Afterward, the essential oil was separated from water using n-hexane. This process was done in a separating funnel, resulting in two fractions. The essential oil dissolved in the n-hexane fraction was collected in a glass beaker, and anhydrous sodium sulfate was added to bind any remaining water mixed with the n-hexane fraction. Then, the n-hexane fraction was distilled again at a temperature of 60 ℃–70 ℃ until no more n-hexane distillate drips out. The resulting essential oil was taken with a micropipette and transferred into a vial [15, 16].

Organoleptic evaluations of bulbs and essential oils. Organoleptic testing is conducted to validate the authenticity of a plant using the five senses. In this study, tests were performed to observe the shape, color, and odor of the bulbs and essential oils of five shallot varieties [17].

Measurement of the refractive index. The refractive index measurement is used to identify and determine the purity and quality of an essential oil [18]. In the present study, measurements were obtained by adding essential oil to a refractometer instrument with a 1:1 n-hexane solvent ratio at a temperature of 20 ℃ [19].

GC-FID fingerprinting analysis. The GC-FID fingerprinting analysis process by Kerdudo *et al*. [20] utilized an HP-1 column (30 m x 250 µm x 0.25 µm). The oven temperature was initially set at 100 ℃ for 13.6 minutes and increased to 180 ℃ at a rate of 3.8 ℃/minute. It was further increased to 250 ℃ at a rate of 20 ℃ /minute. Finally, the temperature was held isothermally at 270 ℃ for 10 minutes. The flow rate of hydrogen gas (H_2) was set at 40 mL/minute, and the airflow rate at the detector was 450 mL/minute. The injection and detector temperatures were set at 275 ℃. Nitrogen served as the carrier gas with a flow rate of 3 mL/minute. A sample of 0.2 µL was injected with a separation ratio of 1:100. Three replications of samples were injected for each variety sample, resulting in a chromatogram profile of the essential oil regarding peak area and retention time.

Chemometric analysis for authentication of shallot variety. Chemometric analysis was performed using MetaboAnalyst 6.0 software, employing techniques such as PCA, HCA, and PLS-DA. Prior to chemometric analysis, chromatogram data were aligned using GCAlignR in RStudio software platform. Then, the resulting data matrix was normalized based on the median and subjected to a log10 transformation. PCA, PLS-DA, and HCA were utilized to visualize groupings and clusters in the chromatogram data, showing the authenticity of Bima Brebes [21, 22].

Results and Discussion

Shallot essential oil extraction. The shallots used in this research were certified shallots classified as extension seeds. In plant certification, there are four types of seed classes: breeder, foundation, stock, and extension seeds. Breeder seeds are highly pure and are owned exclusively by plant breeders or breeding institutions. They are marked with a yellow label and are the source for propagating foundation seeds. Foundation seeds, marked with white labels, are reproduced by the breeder seed and used to propagate stock seeds. Stock seeds, marked with purple labels, are typically propagated by seed breeders and passed down to extension seeds. Extension seeds, marked with blue labels, can be marketed directly to consumers or farmers. Although the seed class used in this study may not have the same level of purity as breeder seeds, it still underwent a certification process by a credible agency or institution to receive the extension seed label. Therefore, this study used shallot seeds guaranteed to be of high quality [23, 24].

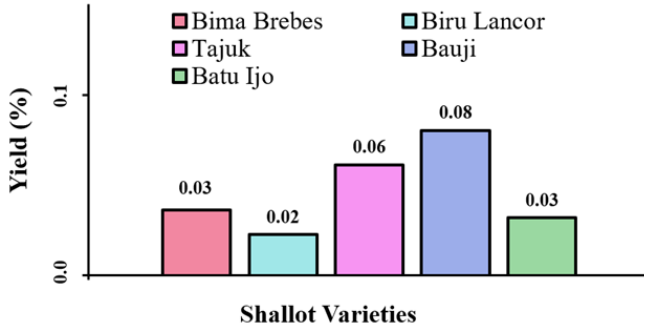

Figure 1. Yield of Essential Oils from Five Shallot Varieties

Moreover, ultrasonic hydrodistillation was used as the extraction method to increase the yield of essential oils. By employing ultrasonic waves, the cavitation phenomenon occurs, damaging the cell walls and facilitating the release of compounds [10]. Additionally, hydrodistillation was performed as it is a commonly used and practical method to obtain essential oils [25]. The essential oil yield obtained from the five shallot varieties (Figure 1) varied, ranging from 0.02% (w/w) to 0.08% (w/w). The Bauji variety yielded the highest amount, whereas the Bima Brebes variety and Batu Ijo variety each yielded approximately 0.03% (w/w), which is about half of the yield of the Bauji variety. The differences in yield could be influenced by genetic variations and geographical conditions, such as the origin and climate of the five varieties [26, 27].

Ferdiansyah *et al*. [10] reported higher production of shallot essential oil at a rate of 0.09% (w/w) using the microwave steam ultrasonic diffusion method. According to Tran *et al*. [28], microwave-assisted hydrodistillation has the advantages of a quicker extraction process and higher recovery of essential oils. Regarding lemongrass essential oils, the present study found that microwave-assisted hydrodistillation yielded a higher amount of 0.35% (v/w) compared to the hydrodistillation method alone that yielded 0.2% (v/w). The efficient heat flow in microwaves enables rapid heating of the entire sample, resulting in an increased speed and quantity of essential oil obtained [29].

Organoleptic test of bulbs and essential oils of shallot varieties. Based on the results of organoleptic tests conducted on five shallot varieties, it was found that the Bima Brebes variety and four other varieties (Table 1) have similar shapes, colors, and odors, making it challenging to distinguish one from the other [7]. However, the Batu Ijo variety was more pronounced owing to its larger shape. These results are consistent with the description provided in the Decree of the Indonesian Minister of Agriculture (KEMENTAN RI) [30]. However, differences were noted regarding color. For example, the organoleptic test results for the Biru Lancor variety indicated a bright red color, whereas the description from KEMENTAN RI states that the color of the Biru Lancor variety bulb is dark purplish red. Additionally, variations were determined in the odor of the shallot bulbs. The description from KEMENTAN RI uses the terms "pungent" or "moderate," whereas the test results indicated "typical shallot aroma." These differences may be influenced by variations in perception during the sensing process; however, overall, the organoleptic test results were consistent with the description by KEMENTAN RI.

The essential oils were evaluated using organoleptic tests, specifically for their form, odor, and color (Table 2). The Bauji variety exhibited a distinct brownish-red color. The essential oil of the Bima Brebes variety had a similar color to that of the Biru Lancor and Batu Ijo varieties, which were yellow, although the brightness levels differed. These observed colors align with the specifications provided by Ferrant Producteur d'Huiles Essentielles (PHE) [31], which state that shallot essential oil should be yellow to brownish in color. However, these results were not sufficient to conclude the authenticity of the Bima Brebes variety, as no significant differences were observed between the varieties. Figures 2 and 3 present the form and color of the bulbs of the five shallot varieties and color of the obtained essential oil.

Refractive index of shallot essential oils. Refractive index measurements can be optimized for qualitative analysis in the authentication process [32]. The refractive index of the five varieties was determined by diluting them with n-hexane in a 1:1 ratio. The obtained values ranged 1.38–1.40 (Figure 4), with the Bima Brebes variety having the highest refractive index value. The Biru Lancor, Tajuk, and Batu Ijo varieties had the same refractive index value of 1.39. Similar refractive index results were obtained in another study [32] wherein refractive index measurements were used to compare the physical characteristics of lavender, sage, and basil essential oils. The results obtained were 1.45, 1.47, and 1.46, respectively.

The present findings seem to be consistent with the specifications provided by Ferrant PHE [31]. Ferrant PHE indicated that the refractive index of pure shallot essential oil (100%) ranges from 1.51 to 1.58. In the present study, we performed a diluted sample (1:1) using n-hexane to determine the refractive index of shallot essential oil. The refractive index of n-hexane is known to be 1.37. Therefore, the refractive index value obtained for diluted shallot essential oil ranged from 1.44 to 1.47, which is still in accordance with the specification range determined by Ferrant PHE. Furthermore, Vârban *et al*. [32] found that diluting lavender, sage, and basil essential oils with canola oil resulted in changes in the refractive index value, tending to produce values according to the contribution ratio of each sample based on the refractive index mixing rules.

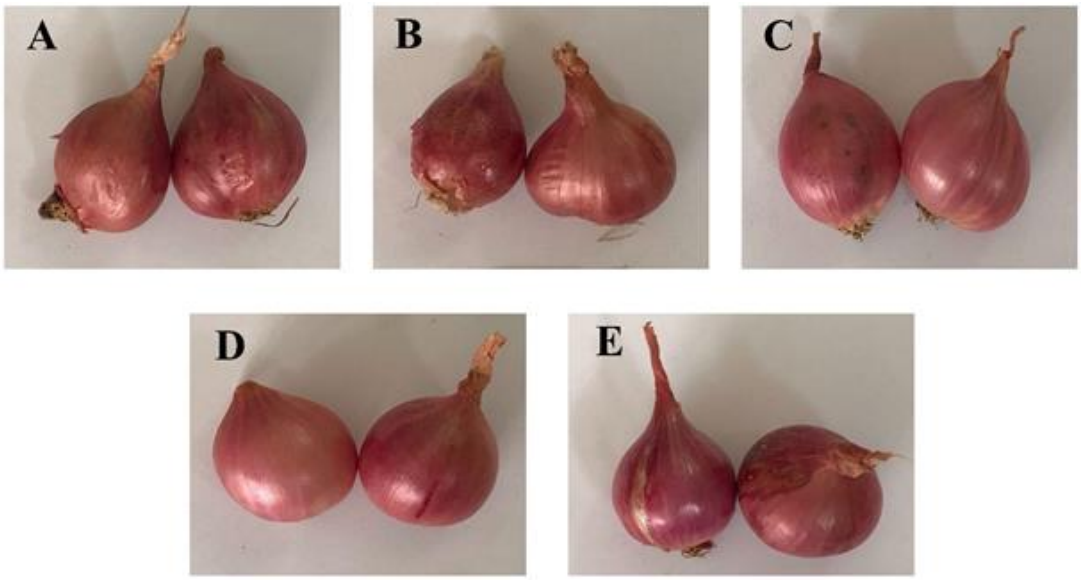

Figure 2. Bulbs of Five Shallot Varieties: (A) Bima Brebes, (B) Biru Lancor, (C) Tajuk, (D) Bauji, and (E) Batu Ijo

Table 1. Organoleptic Evaluations of Shallot Bulbs

Parameter	Shallot variety names				
	Bima Brebes	Biru Lancor	Tajuk	Bauji	Batu Ijo
Shape	Oval round, medium size	Round, medium size	Oval round, medium size	Round, medium size	Round, big size
Color	Bright red	Bright red	Bright red	Bright red	Purplish red
Odor	Typical shallot aroma	Typical shallot aroma	Typical shallot aroma	Typical shallot aroma	Typical shallot aroma

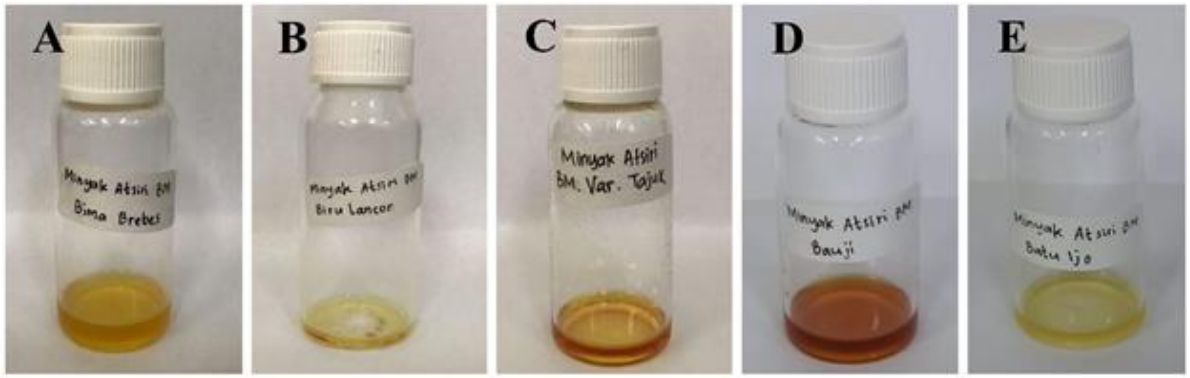

Figure 3. Shallot Essential Oil Color: (A) Bima Brebes, (B) Blue Lancor, (C) Tajuk, (D) Bauji, and (E) Batu Ijo

Table 2. Organoleptic Test Results of Shallot Essential Oil

Parameter	Shallot variety names				
	Bima Brebes	Biru Lancor	Tajuk	Bauji	Batu Ijo
Form	Liquid	Liquid	Liquid	Liquid	Liquid
Color	Dark yellow	Bright yellow	Brownish yellow	Brownish red	Bright yellow
Odor	Typical shallot aroma	Typical shallot aroma	Typical shallot aroma	Typical shallot aroma	Typical shallot aroma

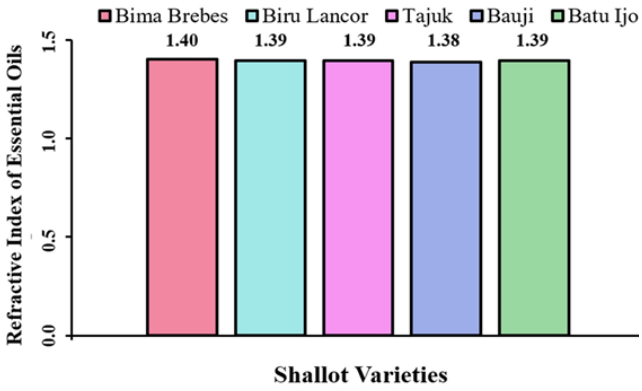

Figure 4. Refractive Index Values of Essential Oils of Shallot Varieties

GC-FID analysis of essential oils of shallot varieties. This study analyzed five different shallot varieties using GC-FID. The resulting data matrix contained approximately 149 peaks. Figure 5 illustrates the chromatogram profiles of the five shallot varieties. The first peak, which appears at around 4–5 minutes with a high-intensity response, corresponds to n-hexane. This organic solvent has a boiling point of 68.70 ℃ , and because the GC temperature is set at 100 ℃ during the first 13.6 minutes, the n-hexane is the first compound to elute from the column during the separation process [33, 34]. The chromatogram profiles reveal several peaks, revealing differences between the varieties. This indicates that the variations in secondary metabolite content can be attributed to the differences in the chromatogram peaks [35]. The Bima Brebes variety exhibits a distinct peak pattern at a retention time of 22–38 minutes, characterizing it from the other four varieties. Similarly, the Biru Lancor variety shows a unique peak at a retention time of 40–45 minutes, which is not observed in the other four shallot varieties. However, further analysis using chemometrics is crucial to validate and visualize the data more effectively [6, 22].

Chemometric analysis for authentication of bima brebes shallot variety. Figure 6 presents a score plot of the PCA results, showing how the five shallot varieties are grouped based on their peak chromatogram profiles. Overall, 149 peaks were used in the PCA plot, accounting for 48.7% of the total variation in principal component (PC) 1 and 2 are 30.3% and 18.4%, respectively. The total variation percentage describes how well the discriminant function can differentiate samples of the same type using the PC characteristics in the model [35]. PC1 and PC2 were chosen for the present analysis because they showed the most differentiation between samples [36]. Notably, the Bima Brebes variety had similar plot scores to the Batu Ijo variety, resulting in overlapping plots. Similarly, the Biru Lancor variety had plot scores close to those of the Batu Ijo variety. In contrast, the Bauji and Tajuk varieties formed a distinct group that was far from the Batu Ijo plot scores, although an overlap was noted. PCA showed that shallot varieties that had almost similar score plot scores or even overlap tend to have comparable characteristics [35]. However, the separation between varieties is unclear; hence, further PLS-DA is required.

The PLS-DA (Figure 7a) demonstrates a clearer distinction between the different varieties compared to PCA. PLS-DA is a classification method that combines the partial least squares approach with discriminant analysis [21]. The total variance obtained was 45.2% (component 1: 26.9%; component 2: 18.3%). The plot scores indicate that the five varieties form distinct groups, and the Bima Brebes variety is distinct from the others. Another crucial parameter in PLS-DA is the Variable Importance in Projection (VIP) score. In this case, the retention time peak with a VIP score of >1 was considered a significant feature in grouping the five shallot varieties [21]. Figure 7b displays the 25 peaks at specific retention times that have the most influence on grouping.

An issue in PLS-DA is overfitting. To address this, the performance of the PLS-DA model should be validated using cross-validation (CV) and permutation tests. CV allows for the evaluation of the predictive ability and accuracy of classification models on random response data. In Figure 8a, the results of a fivefold CV are presented, which yield a PLS-DA model with a prediction accuracy of 0.783 (R^2 = 0.988; Q^2 = 0.869). Figure 8(B) presents the permutation test results; a permutation test was conducted for 20 iterations of the PLS-DA model. The test yielded a p-value of <0.05, indicating that the permutation data distribution is close to the accurate distribution. Consequently, the PLS-DA model used in this analysis is not overfitting and can be used for predictions [21, 36].

The dendrogram for HCA is generated using the Euclidean distance and a Ward clustering algorithm. Fifteen samples were grouped into clusters based on their varieties (Figure 9). The Bima Brebes variety was observed to have the highest similarity to the Batu Ijo variety, whereas the Bauji variety had the lowest similarity to the Bima Brebes variety [21]. In this study, the chemometric analysis visualized chromatogram data for the authentication of Bima Brebes shallots.

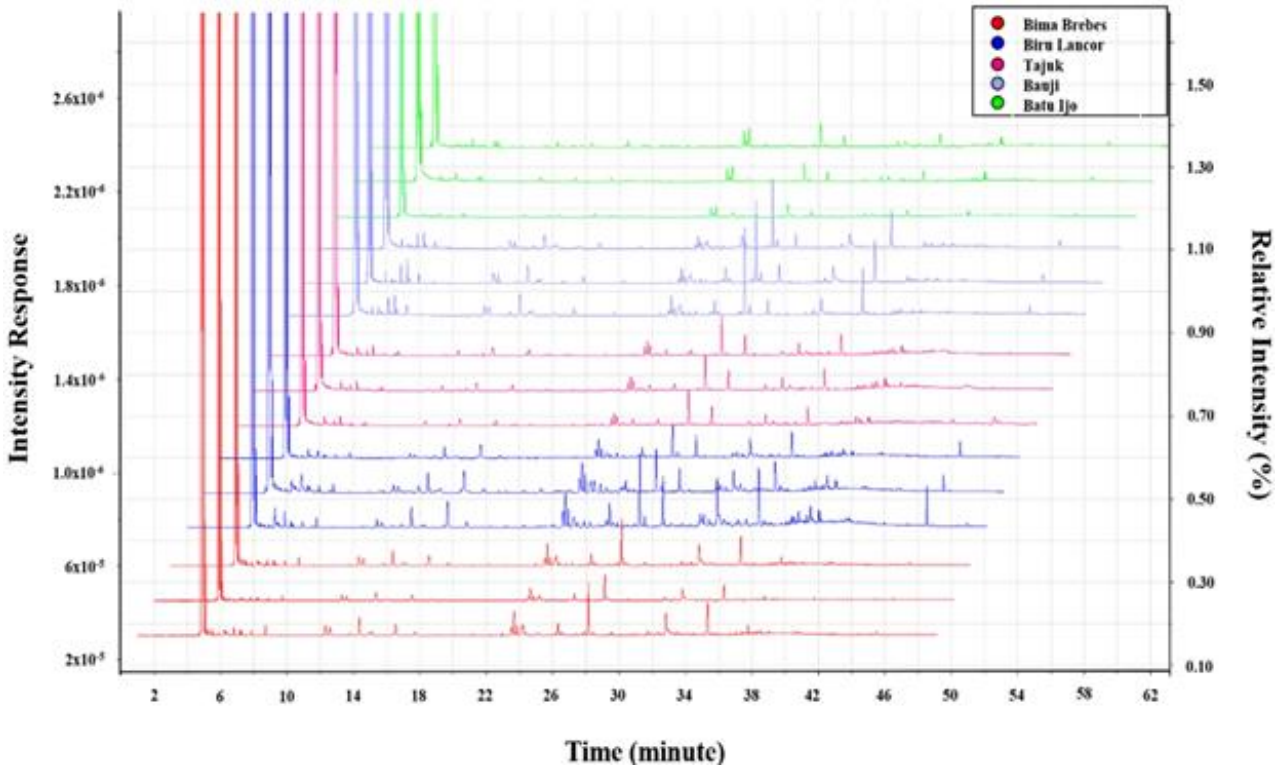

Figure 5. Chromatogram Profile of Essential Oils of Five Shallot Varieties

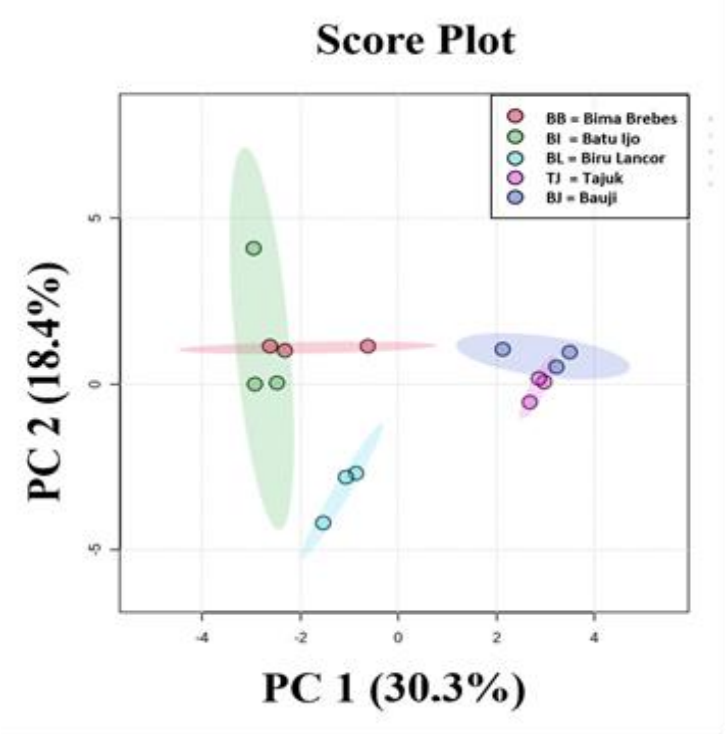

Figure 6. PCA Score Plot of Shallot Varieties

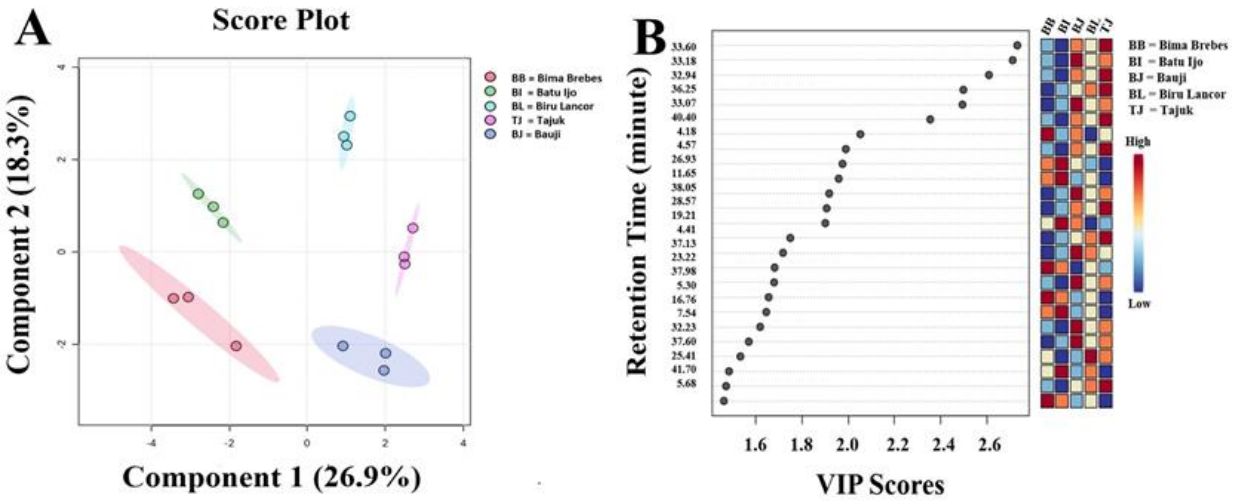

Figure 7. (A) Score Plot and (B) VIP Scores PLS-DA of Shallot Varieties

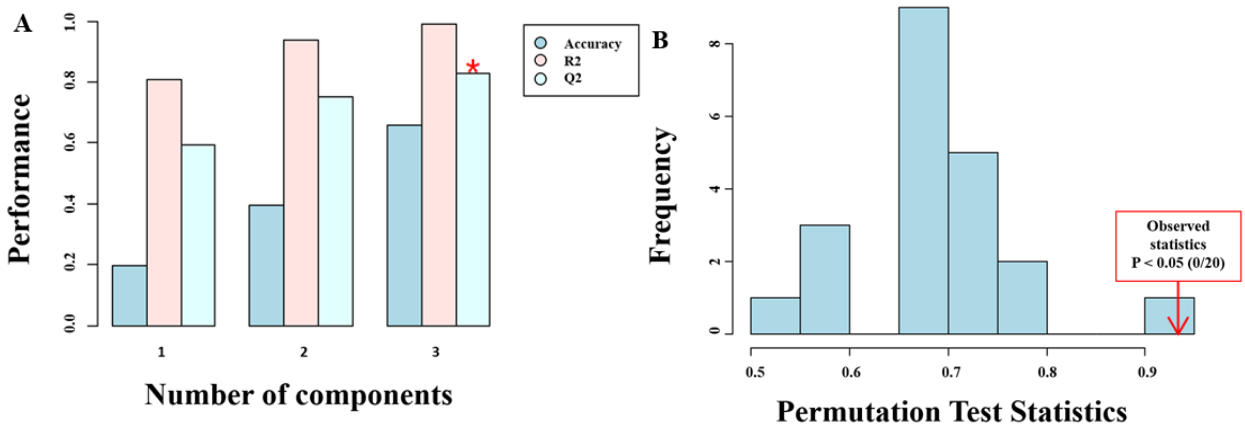

Figure 8. (A) Results of CV Analysis and (B) Permutation Test on the PLS-DA Model of Shallot Varieties

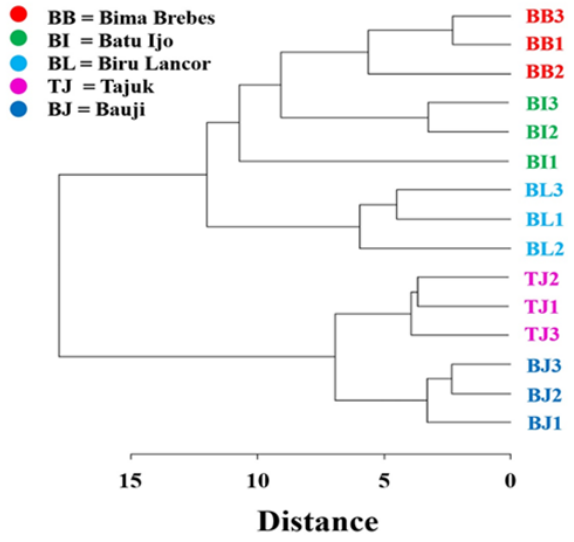

Figure 9. Dendrogram of HCA Results of Five Shallot Varieties

Conclusion

This study aimed to determine the authenticity of Bima Brebes shallots using chemometrics to analyze the GC-FID fingerprinting chromatogram profile. Using PLS-DA and HCA, five shallot varieties were grouped and differentiated. This analysis method can be used for authenticating Bima Brebes shallots and as reference for future research in food ingredient authentication. However, the study did not identify biomarkers that significantly contribute to the grouping process. Therefore, future research should incorporate mass spectrometry detectors in conducting metabolomic analysis and identifying biomarker compounds that play a crucial role in grouping.

Acknowledgements

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