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Identification of some coffee leaf taxa using fluorescence spectroscopy and chemometrics

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ABSTRACT: Analytical techniques for identification of coffee taxa are essential for plant breeding and quality control of products. Rapid technique for discrimination of coffee taxa based on the fluorescence signals from their leaf extracts was introduced. Five different coffee taxa: *Coffea liberica*; *Coffea congensis*; *Coffea arabica* var. Geisha, a spontaneous hybrid of *C. Arabica* and *Coffea canephora* (Hibrido de timor), a hybrid of Hibrido de timor, and *C. arabica* var. *Cattura*; were investigated based on their fluorescence signals. The individual taxa present different fluorescence spectra. The spectra obtained from the excitation wavelengths at 300, 330, 390, 420, and 450 nm; and emission wavelengths in the range of 500–790 nm were selected for principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA). It was found that fluorescence signals with excitation wavelength at 300 nm had been successfully implemented for rapid clustering and identification of some coffee taxa. The PCA score plot presenting natural clustering of data obtained from the fluorescence spectra tended to agree with the data of chemical contents based on antioxidant activity, total phenolic content, and total flavonoid content. The Q^2 and R^2 calculated via leave-one-out cross-validation (LOOCV) of model obtained from processing of the PLS-DA were 0.6 and 0.8, respectively. It means that the model has potential for the categorization of coffee taxa based on their leaf extracts without any chemical treatments.

KEYWORDS: fluorescence spectroscopy, coffee leaves, coffee taxa, taxonomic identification, chemometric analysis

INTRODUCTION

Coffee species and varieties are important factors that affect coffee qualities. The identification of coffee is very important for the exploitation in coffee plant breeding and quality control of coffee products as well as the development of taste and favor diversity. There are more than 120 varieties of coffee, but the two most popular are *C. arabica*, commonly known as Arabica, and *C. canephora* known as Robusta [1]. Many scientific reports on taxonomic analyses of coffee are available on *C. arabica* and *C. canephora*. However, there are limited information of other coffee species and their varieties in the scientific literature. More information on genetic diversity of coffee would be beneficial to the development of coffee taste and favor diversity in the future. The discrimination of coffee taxa has been studied using morphological-based techniques [2], molecular DNA analysis [3], and traditional laboratory-based chemical methods [4]. In addition, there are many chemical analysis techniques applied for the classification of plant extracts obtained from various varieties, such as high-performance liquid chromatography-diode array (HPLC-DAD) [5], HPLC-diode array detector-time-of-flight-mass spectrometry (HPLC-DAD-TOF-MS) [6], and nuclear magnetic resonance spectroscopy (NMR) [7]. These techniques could improve performance of discrimination and classification by coupling with chemometric analysis. However, these methods require laborious and time consuming analytical workflow. Several spectroscopic techniques are successfully applied for taxonomic identification of living things due to their rapidity, cost effectiveness, and non-tedious sample preparation [8, 9]. Recently, the

combinations of FT-NIR spectroscopy and chemometrics, such SIMCA analysis, have been successfully used to identify coffee leaves taxa [10]. Besides, Laser-Induced Breakdown Spectroscopy and chemometrics have been used to identify coffee varieties from their beans [11]. Fluorescence spectroscopy is one of the rapid techniques implemented for taxonomic study in a wide range of organisms such as bacteria [12–15], microalgae [16], fungi, and plants [17–19]. Due to the presence of various autofluorescent molecules, such as nicotinamides (NADPH, NAD), pterins, phenols (hydroxycinnamic acid), alkaloids, flavins (FAD, FMN), flavonoids, terpenoids, polyacetylenes, isoquinolines, chlorophylls, anthocyanins, and anthocyanidins [20], leaves of typical green plants provide numerous differences of fluorescence spectra. Therefore, fluorescence spectra from leaves were widely used for fingerprint construction and taxonomic identification of plants. There are abundances of various fluorescent secondary metabolites in coffee leaves, such as chlorogenic acid (5-caffeoyl-quinic acid, 5-CQA)), mangiferin (C2-β-D-glucoside-1,3,6,7-tetrahydroxyxanthen-9-one), and caffeine (1,3,7-trimethylxanthine) [20]. However, the literature contains no report on the use of fluorescence spectroscopy for coffee taxonomic study. Identification of coffee taxa based on their leaves is interesting because leaves could be collected easily all year round and no limitation of fruiting season.

In the present study, cost effective, rapid, and non-destructive fluorescence spectroscopy combined with chemometrics, such as principal component analysis (PCA) and partial least squares - discriminant analysis (PLS-DA), were used for the identification of some coffee leaf taxa obtained from the same planting area [21]. The algorithm of PCA, an unsupervised method, was processed to understand the natural clustering of the fluorescence signals of coffee leaf extract without referring to class labels. Because coffee leaf contains various fluorescent secondary metabolites presenting biological activities, such as chlorogenic acids (CGA) and mangiferin [20, 22], the PCA results obtained from fluorescence spectra were compared with the PCA data from antioxidant activity, total phenolic content, and total flavonoid content of coffee leaf samples. Hierarchical cluster analysis (HCA) in the form of dendrogram was also used to demonstrate the interrelationships between samples. Then, the algorithm of PLS-DA, a supervised method, was performed to construct a model for classification of coffee taxa.

MATERIALS AND METHODS

Chemicals and reagents

Methanol and sodium hydroxide (97%) were purchased from RCI Labscan Company (Thailand). The L(+)-ascorbic acid standard was obtained from POCH (Poland). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the (±)-catechin hydrate primary reference standard was purchased from Sigma Aldrich (US). The gallic acid monohydrate standard was purchased from Fluka (Spain). Sodium carbonate anhydrous was obtained from Ajax Finechem (Australia) and aluminium chloride hexahydrate (AR Grade) was ordered from QReC (New Zealand). Sodium nitrite (98%) was obtained from LOBA CHEMIE (India). Folin-Ciocaltue's phenol reagent was purchased from MERK (Germany). All reagents and chemicals used were of analytical grade.

Sampling and sample preparations

The coffee leaves of four different coffee taxa (*C. liberica*, *C. arabica* var. Geisha, a spontaneous hybrid of *C. arabica* and *C. canephora* (Hibrido de timor) named Hibrido de timor hereafter, and a hybrid of Hibrido de timor and *C. arabica* var. *Cattura* (named H306/1 ML1/1 hereafter) were collected for chemical content analyses (antioxidant activity, total phenolic content, and total flavonoid content). The same four taxa plus *C. congensis* were collected for fluorescence analysis. Those coffee leaves were obtained from Mae Lod Royal Agricultural Research Station (the Royal Project Foundation), Chiang Mai, Thailand in mid-December (during fruit ripening period) 2018 and 2019.

The sampling of coffee leaves was carried out using the minor modified method of Sousa et al [23] and Tamimi et al [24]. The five branches were selected by counting down from the top of the vertical to the 8th to 12th lateral branch. Then, the recently matured leaves from these laterals, usually the 3rd or 4th pair back from the branch tip, were picked. These leaves should be full-sized with the same color and texture. Three coffee trees of each taxon were marked, and at least 15 leaves were collected per tree.

All leaf samples were cleaned, dried at 60 °C in an oven for 3 h, and then ground into powder using a blender. The extraction was performed in triplicate by soaking 0.1 g coffee leaf powder in 1.5 ml of methanol for 12 h followed by shaking the sample solutions for 3 h at 150 rpm in a water bath shaker (Wisd laboratory instrument, Korea). Then the solutions were filtered into 5 ml-volumetric

flasks, adjusted the volume to 5 ml, and stored in a refrigerator for further analyses.

Antioxidant activity assay

The DPPH radical scavenging activity of the methanolic extracts obtained from coffee leaf samples were carried out using the minor modified method of Saw et al [25]. Solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH, 0.3 mM) was prepared in methanol. Aliquots of 200 µl of sample extracts were mixed with 1 ml of DPPH solution. Then, the solution's volume was adjusted to 4 ml by ultra-pure water (Millipore Milli-Q™ Reference Ultra-pure Water Purification System), mixed by a vortex mixer (VM-10 model, Wisd laboratory instrument, Korea), and then incubated for 30 min in a dark room. Standard solutions of ascorbic acid in the concentration range of 0–0.018 mM were prepared. The absorbance of the solutions was measured at 515 nm [26] using a visible spectrophotometer (USB4000, Ocean Optics, USA). The DPPH radical scavenging activities of all extracts were calculated using the ascorbic standard curve. The results were presented as milligram ascorbic acid equivalent per gram dried coffee leaf weight (mgAAE/g dried weight).

Total phenolic content

Analyses of the total phenolic content in coffee leaf extracts were carried out using the method proposed by Haile et al [27] with minor modification. Briefly, Folin-Ciocalteu reagent (10% w/v) dissolved in methanol was used as reagent. The leaf extract sample (150 µl) was mixed with the Folin-Ciocalteu reagent (125 µl) and incubated for 5 min. Then, the solution was mixed with 1.25 ml of Na_2CO_3 (7% w/v) and adjusted the volume by using ultra-pure water to 3 ml. The solution was mixed and then incubated in a dark room for 1 h. After that, the absorbance of the solution was measured at 765 nm using a visible spectrophotometer. Different concentrations of gallic acid standard solution were prepared in the range of 0 to 100 ppm. The total phenolic content was presented as milligram gallic acid equivalent per gram dried coffee leaf weight (mgGAE/g dried weight).

Total flavonoid content

Analyses of total flavonoids content in coffee leaf extracts were carried out using the method proposed by Phuyal et al [28] with minor modification. The leaf extract sample (200 µl) was mixed with 0.55 M of $AlCl_3$ (100 µl) and, then, 3.0 M of $NaNO_2$ (50 µl). After that, 2.5 M NaOH (250 µl) was added into the mixture solution. The solution volume was adjusted to 2 ml by ultra-pure water. The solution was mixed and incubated for 30 min in a dark room. The absorbance of the solution was measured at 510 nm by using a visible spectrophotometer. The calibration standard curve was prepared using catechin standard in the concentration range of 0 to 100 ppm. The flavonoids content was presented as milligram catechin equivalent per gram dried weight (mgCE/g dried weight).

Measurement of fluorescence spectra

A fluorescence spectrophotometer (LS55 model, PerkinElmer, USA) equipped with a xenon lamp and a photomultiplier detector was used for fluorescence spectrum analysis of the leaf extract samples. The excitation wavelength for detecting fluorescence signal of the studied solution was set from 270 to 460 nm. The emission wavelength was monitored in the range of 500 to 800 nm. All the measurements were performed in a standard fluorescence 96-well plate with a scan rate at 800 nm/min and 10 nm bandwidths for the emission and excitation monochromators.

Statistical and multivariate analysis

The spectra of 3D-fluorescence contour plot of all sample extracts were investigated for selecting the excitation wavelengths and the range of emission wavelength. The excitation wavelengths producing different fluorescence intensities in the range of 500–790 nm were selected for further statistical and multivariate analyses. Then, the data were filtered using interquartile range before processing. Normalization was performed by the sample median method. The fluorescence intensities obtained from the selected excitation wavelengths of all extracts were processed by a PCA to study the natural clustering of the samples. Statistical analyses were performed by mean of the MetaboAnalyst® 4.0 online program running by R-script chemometrics [29]. The PCA clustering result obtained from the data set of fluorescence spectra was compared with that obtained from the data set of chemical contents based on antioxidant activity, total phenolic content, and total flavonoid content of coffee leave samples.

Then, hierarchical cluster analysis (HCA) in the form of a dendrogram was also used to study the interrelationships between samples. Two important parameters were considered for performing HCA. The first one was similarity measure including Euclidean distance, Pearson's correlation, and

Spearman's rank correlation. The other parameter was clustering algorithms including average linkage, complete linkage, single linkage, and Ward's linkage.

Finally, partial least squares - discriminant analysis (PLS-DA) was processed to develop a model for classification of the membership. Leave-one-out cross validation (LOOCV) was used for cross validation. The quality assessments of Q^2 and R^2 calculated via cross-validation (CV) were used for the estimation of the qualitative measurement of the model consistency between the predicted and the original data [30]. In addition, Variable Importance in Projection (VIP) values obtained from sum of squares of the PLS loadings, which consider the amount of explained Y-variation in each dimension [31], were also calculated.

RESULTS AND DISCUSSION

Multivariate analysis of antioxidant activity, total flavonoid content, and total phenolic content

The chemical contents, including antioxidant activity, total flavonoid content, and total phenolic content, found in four coffee leaf extracts (*C. liberica*, *C. arabica* var. Geisha, Hibrido de timor, and hybrid H306/1 ML1/1) are shown in Fig. 1. The chemical contents found in individual coffee leaf taxa seem to be different. The 2D-scores plot between the selected principal components (PCs) obtained from the PCA using the chemical contents of the samples are shown in Fig. 2. The result showed that the *C. liberica*, *C. arabica* var. Geisha, and Hibrido de timor samples could be clustered clearly according to their chemical contents. The literature reported that the coffee beans of *C. liberica* presented higher chemical contents (based on the result of the total phenol assay, the DPPH assay, and the ferric reducing antioxidant power (FRAP) assay) than that of *C. robusta* and *C. arabica* [32]. However, the H306/1 ML1/1 (hybrid coffee taxon) could not be clustered clearly from the *C. liberica* and *C. arabica* var. Geisha. Therefore, the data set from other chemical factors might be required for classification of hybrid coffee taxa.

Fluorescence spectral characteristics of coffee leave extracts

Fluorescence spectra with emission spectra in horizontal axis and excitation spectra in vertical axis of the methanolic coffee leaf extracts (*C. liberica*, *C. congensis*, *C. arabica* var. Geisha, Hibrido de timor, and H306/1 ML1/1) are shown in Fig. 3. The contour line patterns of each coffee leaf extract were different. These fluorescence results might be due to the differences of concentration of chemical composition in the extract samples as reported by Yisak et al [32]. Besides, it could be observed that excitation wavelength at 420 nm provided high intensity of fluorescence signal. The fluorescence intensities of each sample obtained from excitation

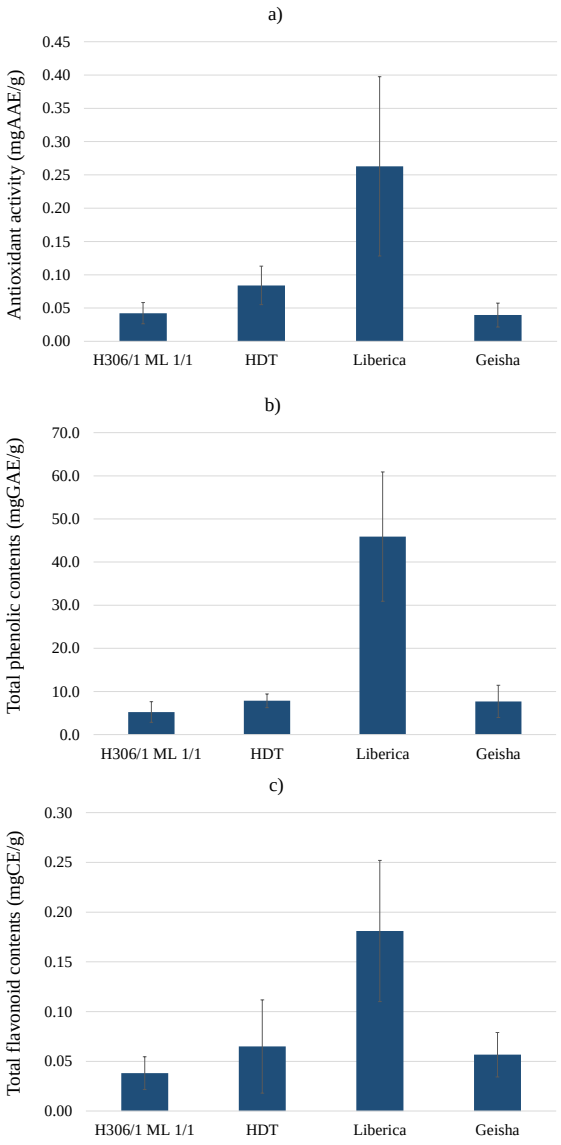

Fig. 1 Antioxidant activities (a), total phenolic content (b), and total flavonoid content (c) found in four coffee leaf extracts: *C. liberica* (Liberica), *C. arabica* var. Geisha (Geisha), Hibrido de timor (HDT), and a hybrid H306/1 ML1/1.

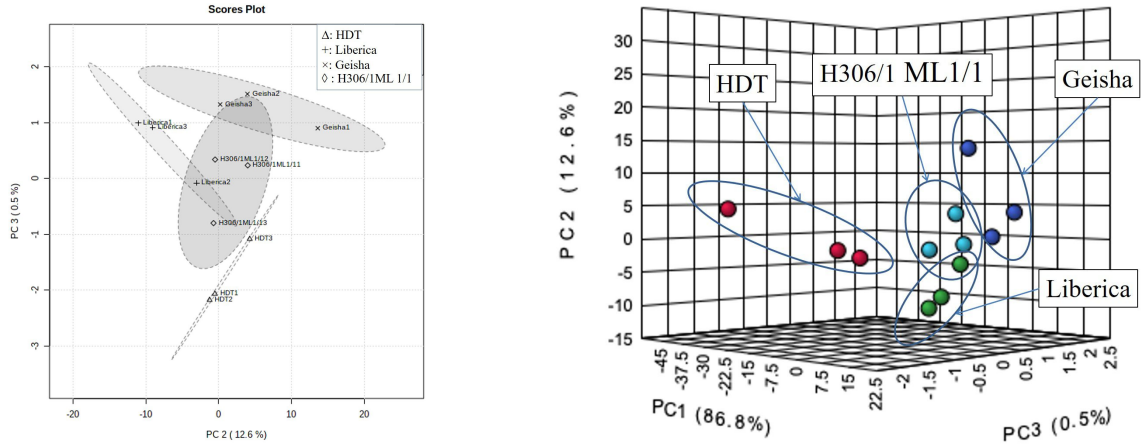

Fig. 2 The 2D (a) and 3D-scores plot (b) between the selected principal components (PCs) obtained from the principal component analysis (PCA) according to the chemical contents of the samples: *C. liberica* (+), *C. arabica* var. Geisha (×), Hibrido de timor (△), and hybrid H306/1 ML1/1 (◇).

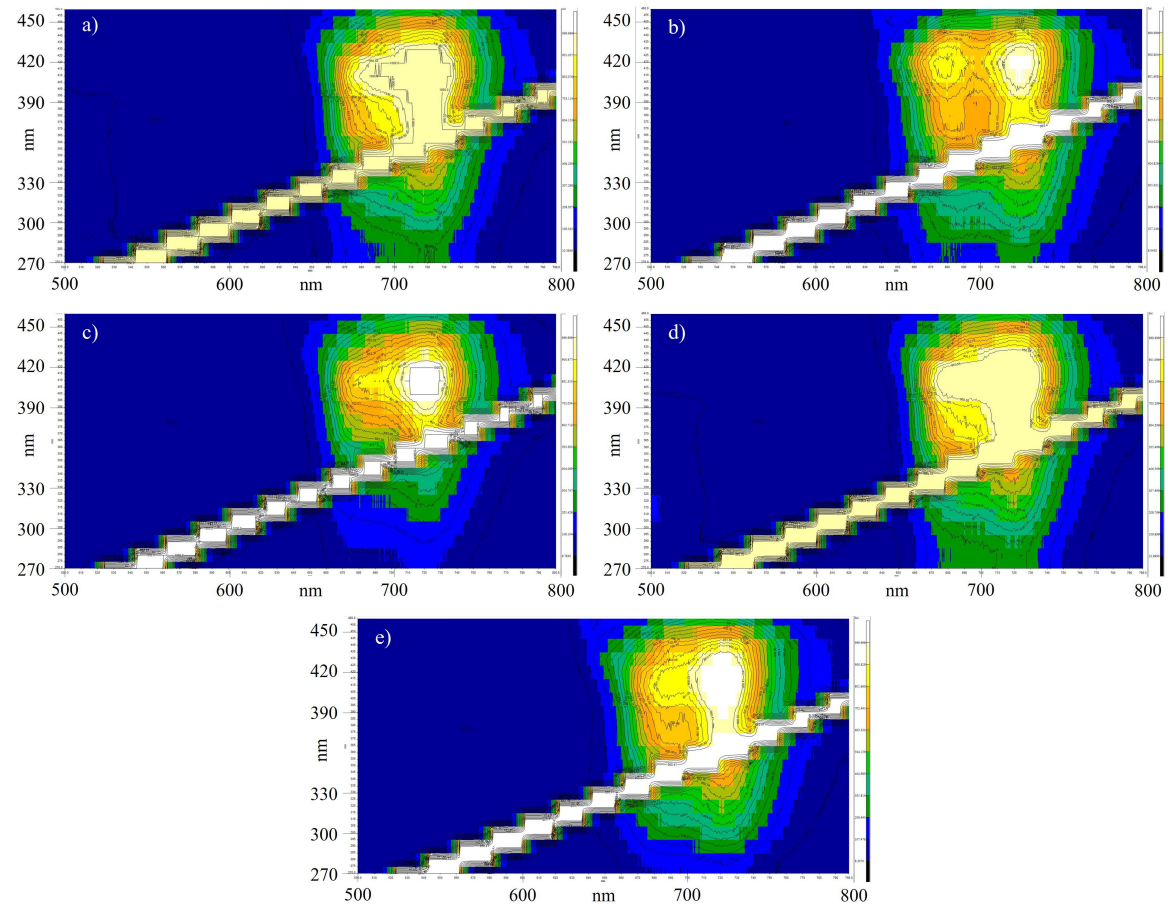

Fig. 3 Contour plots of the fluorescence spectra with emission spectra in horizontal axis and excitation spectra in vertical axis of the methanolic coffee leaf extracts: *C. liberica* (a), *C. congensis* (b), *C. arabica* var. Geisha (c), Hibrido de timor (d), and a hybrid H306/1 ML1/1 (e).

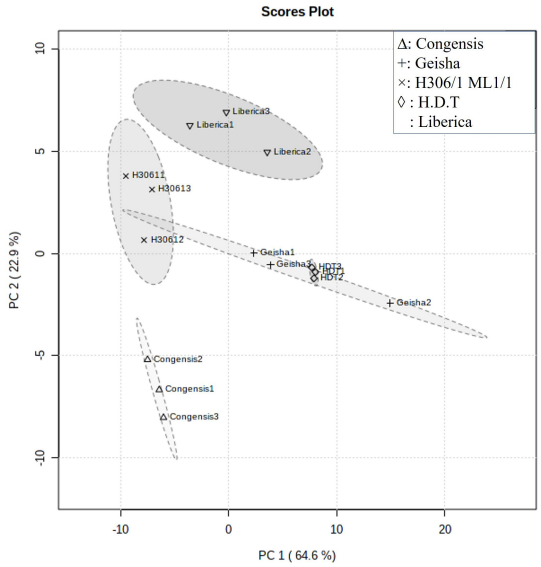

Fig. 4 The scores plot between the selected principal components (PC1 × PC2) of raw spectra obtained from using excitation wavelengths at 300 nm: *C. liberica* (▽), *C. congensis* (△), *C. arabica* var. Geisha (+), Hibrido de timor (◇), and hybrid H306/1 ML1/1 (×).

wavelength at 300, 330, 390, 420, and 450 nm and the emission range from 500 to 790 nm were selected to perform multivariate analysis for distinguishing coffee taxa, based on the differences of fluorescence intensity.

Multivariate analysis of fluorescence spectra

A PCA of the selected fluorescence intensities was processed via the MetaboAnalyst® 4.0 (web online) to examine the natural clustering of the coffee taxa. The fluorescence intensities obtained from the combination of emission wavelengths and excitation wavelengths were assigned as data sets. The scores plot between the selected PCs of individual spectra obtained from using several excitation wavelengths was considered. It was found that *C. liberica*, *C. arabica* var. Geisha, hybrid H306/1ML 1/1, and *C. congensis* could be clustered clearly using fluorescence intensity from the excitation wavelength at 300 nm, except Hibrido de timor coffee that showed overlapping with *C. arabica* var. Geisha (Fig. 4). Using fluorescence spectra obtained from excitation wavelength at 300 nm tended to separate the data set as compared with the results of chemical contents, which could separate only three coffee taxa.

The model using fluorescence intensities obtained from excitation wavelength at 300 nm of individual coffee leaf taxa could be performed using partial least squares-discriminant analysis (PLS-DA). Variable Importance in Projection (VIP) values obtained from sum of squares of the PLS loadings and performance of classification are shown in Fig. 5. The VIP values presented that the fluorescence intensities obtained from emission wavelengths from 580 to 620 nm had influence on classification of coffee taxa. The results might be affected by alkaloids found in coffee, such as caffeine, theobromine, and trigonelline, as their maximum UV-visible excitation bands were obtained at 272.89 nm, 272.73 nm, and 264.59 nm, respectively [32, 33]. The amount of those alkaloids in green coffee beans is influenced by numerous factors such as coffee variety, genetic properties of the cultivars, and environment [34]. Moreover, secondary metabolites in coffee leaves that might influence the clustering result are chlorogenic acid (5-CQA) and mangiferin (phenolic compounds) because both compounds exhibit a broad spectral range with peaks at about 425 and 520 nm, respectively. Other compounds of influence might be flavins, flavonoids, some terpenoids, polyacetylene, isoquinoline, and some alkaloids because these compounds emit fluorescence in the red region of the visible spectrum [20]. The quality assessments of Q^2 and R^2 calculated via Leave-one-out cross-validation (LOOCV) were 0.6 and 0.8, respectively (Fig. 5). The red star in Fig. 5 indicates the best classifier. The literature suggests that R^2 value of 0.67 is substantial model [35]. Although Q^2 has no standard of comparison or critical value for inferring significance, an empirically inferred value of $\geqslant 0.4$ is acceptable for a biological model [36]. The Q^2 is close to the R^2 when the PLS built on a training set is applied to a test set. It means that PLS model works independently of the specific data used to train the PLS model. Therefore, there is a tendency to use the fluorescence spectra of methanolic-coffee leaf extracts obtained from the excitation wavelength at 300 nm, and the emission range from 500 to 790 nm for identification of coffee taxa.

An optimized dendrogram of raw fluorescence spectra of coffee leaf extracts (emission wavelength at 300 nm), using Euclidean distance as similarity measure and average linkage as clustering algorithm is shown in Fig. 6. The groups of *C. liberica*, hybrid H306/1ML 1/1, and *C. congensis* were well separated. However, the groups of *C. arabica* var. Geisha and Hibrido de timor were overlapped. The natural clustering of the samples presented as a den-

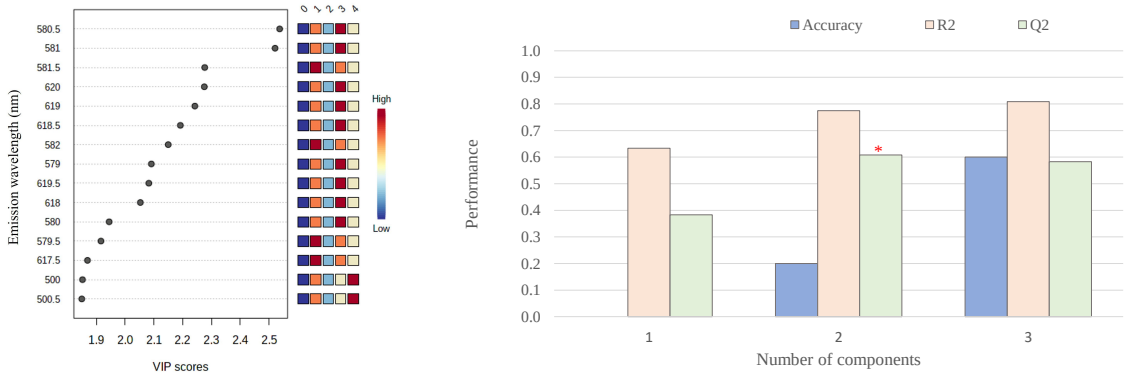

Fig. 5 Variable importance in projection (VIP) values obtained from sum of squares of the PLS loadings (a) and performance of classification; (b) *C. congensis* (group 0), *C. arabica* var. Geisha (group 1), hybrid H306/1 ML1/1 (group 2), Hibrido de timor (group 3), and *C. liberica* (group 4).

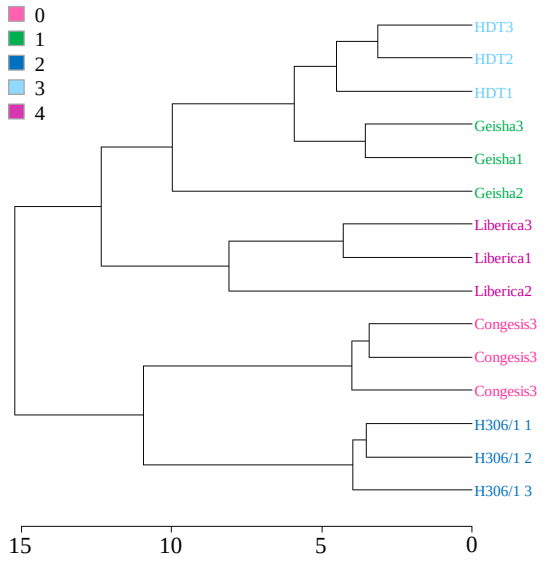

Fig. 6 Clustering result shown as dendrogram (distance measure using Euclidean and clustering algorithm using average): *C. congensis* (0), *C. arabica* var. Geisha (1), hybrid H306/1 ML1/1 (2), Hibrido de timor, HDT (3), and *C. liberica* (4).

drogram agrees with the PCA scores plot between the selected principal components (PC1 × PC2) of raw spectra obtained from excitation wavelength at 300 nm. This result might be due to genetic factors. Both *C. arabica* var. Geisha and Hibrido de timor coffee originated from same Arabica variety.

Multivariate analysis of both the chemical contents and the fluorescence spectra obtained from coffee leaf extracts could be applied for study of the natural clustering in data. However, the fluorescence spectroscopy coupled with chemometric techniques for data clustering and identification is preferred because the analytical methods are chemical and time consuming. On the other hand, the fluorescence technique requires only a few easy steps of sample extraction. In addition, fluorescence spectrum is represented from various autofluorescent metabolites in sample. It is proved to be simple, high sensitivity, and less sample consumption [37].

CONCLUSION

Multivariate analysis of the data of chemical contents and fluorescence spectra for simple classification of some coffee leave taxa extracted with methanol was studied. Principal component analysis according to chemical contents obtained from antioxidant activity, total flavonoid content, and total phenolic content could cluster clearly between Hibrido de timor, *C. liberica* and *C. arabica* var. Geisha. The PCA score plot of fluorescence spectra obtained from excitation wavelength at 300 nm also separated clearly between *C. liberica*, hybrid H306/1ML 1/1, and *C. congensis*. It agrees with the result of the hierarchical cluster analysis presented as a dendrogram. Due to the PLS model works independently on specific data, there is a tendency to use the fluorescence spectra of methanolic extracts obtained from the excitation wavelength at 300 nm and the emission range from 500 to 790 nm for coffee classification study. Fluorescence spectroscopy coupled with chemometric techniques is recommended as screening methods for coffee taxa identification because it requires a few easy steps of sample extraction. It is proved to be simple, high sensitivity, and less sample consumption.

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