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Recognition of Sesquiterpenoids and Piperidine Alkamides as Two Discerning Metabolite Classes in the Fruits of *Piper nigrum* 'Semongok Aman'

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ABSTRACT

The Malaysian Pepper Board (MPB) has recommended the plantation of three over seven *Piper nigrum* L. cultivars, owing to their beneficial agronomic traits. Currently, distinction between the cultivars is assessed based on morphological characters. The MPB has also proposed the concept of monovarietal farm, which is believed to have the potential of strengthening the quality of pepper in the global market. However, there remains a need for a fair assessment of the specialised metabolites' variation among *P. nigrum* cultivars in search of a cultivar with distinctive metabolites profile, which may be the most suitable candidate for the application of such concept. We hereby describe revised protocols aimed at minimising the oxidation of the fruits of five *P. nigrum* cultivars and reducing the experimental run time that allowed utilisation of the same samples in GC-MS and ^{1}H-NMR metabolomics. Subsequently, feature-based molecular network (FBMN) was used to verify the patterns observed in the principal component analysis (PCA) of the ^{1}H-NMR and GC-MS data. PCA of both datasets revealed that the clustering pattern of the five cultivars paralleled the origin of their parent plants, with the genetically more similar cultivars, 'Kuching', 'Semongok Emas', 'India', and 'Yong Petai', being closer to each other compared to 'Semongok Aman'. 'Semongok Aman' was found to contain a higher abundance of the sesquiterpenoids germacrene B and γ-elemene, as well as the piperidine alkamides piperine and its isomers. FBMN further highlighted the higher abundance of the two metabolite classes in the fruits of 'Semongok Aman'. 'Semongok Aman' might be a suitable cultivar for the implementation of monovarietal pepper farm concept owing to its distinctive metabolite profile.

Keywords: Alkamides, cultivar, FBMN, metabolomics, *Piper nigrum*, sesquiterpenoids

INTRODUCTION

Recognised as the 'King of Spices', black or white pepper, the fruit of *Piper nigrum* L., is traded globally for its culinary and medicinal uses (Ashokkumar *et al.*, 2021; Takooree *et al.*, 2019). In Malaysia, the state of Sarawak is the largest pepper producer and boasts the prestigious geographical indication (GI)-certified Sarawak pepper, which is responsible for about 98% of the country's pepper harvest (Entebang *et al.*, 2021). Sarawak pepper is a final product obtained from the processed fruits of several cultivars of *P. nigrum* grown in Sarawak. Presently, it consists of a mixture of the fruits of different cultivars, as the concept of monovarietal pepper cultivation is not currently practised in Malaysia (Chen *et al.*, 2018).

The differences between the cultivars are discerned by manual assessment of a set of six diagnostic morphological characters, which include the qualitative and quantitative traits of leaves, inflorescences, fruits, seeds, and shoot

tips (Chen & Tawan, 2020a). Notwithstanding the morphological diversity and distributional variation of the cultivars, the Malaysian Pepper Board (MPB) has maintained its recommendation for the cultivation of 'Kuching', 'Semongok Emas', and 'Semongok Aman' (Fong & Liang, 2011; Gaweng & Lai, 2017; Johny *et al.*, 2020; Entebang *et al.*, 2021). This recommendation is based on the results of extensive yield verification studies that demonstrated the superiority of the three cultivars over seven other non-recommended cultivars in several positive agronomic aspects. These include consistent percentage yields of green, black, and white pepper, uniform fruit ripening, and increased resistance to pest infestation and fatal diseases (Fong & Liang, 2011; Gaweng & Lai, 2017).

Of the three cultivars, 'Semongok Aman' demonstrates superior chemical properties, having the highest contents of piperine, oleoresin, and volatile and non-volatile oils in its fruits (Fong & Liang, 2011; Gaweng & Lai, 2017). Nevertheless, a comparative analysis of the piperine content and essential oil composition in the fruits of the ten cultivars revealed the highest piperine content was in a non-recommended cultivar, while six out of seven non-recommended cultivars had the highest amount of certain monoterpenoids and sesquiterpenoids (Chen & Tawan, 2020b). Although studies have examined the correlation between pharmacological properties and specialised metabolites (Ashokkumar *et al.*, 2021; Luca *et al.*, 2021; Wang *et al.*, 2021) and the variation in specialised metabolite profiles among different origins (Ahmad *et al.*, 2020; Hashimoto *et al.*, 2021; Jaidee *et al.*, 2022; Liang *et al.*, 2021; Rivera-Pérez *et al.*, 2021) and cultivars (Barata *et al.*, 2021) of *P. nigrum* fruits, data on metabolite profiles of *P. nigrum* fruits from recommended and non-recommended cultivars grown in a single farm are currently lacking.

Therefore, the aim of the present research was to study the variations in metabolite fingerprints and profiles of fruits from three recommended and two non-recommended *P. nigrum* cultivars grown on a farm in Sarawak. Since previous studies have demonstrated that polyphenol oxidase (PPO) in the fruit's skin is the enzyme responsible for blackening of freshly harvested green *P. nigrum* fruits (Bandyopadhyay *et al.*, 1990; Gu *et al.*, 2013), which can obfuscate a fair comparative analysis between the cultivars, the specific objectives of the present research were to: (1) establish a revised protocol for the preparation of plant material and extracts for reliable comparative analysis of metabolite fingerprints and profiles *P. nigrum* fruits of the five selected cultivars; (2) analyse the metabolite classes contributing to the observed variation; and (3) determine the cultivar exhibiting the most distinctive metabolite fingerprints and profiles. The findings may shed light on the application of monovarietal cultivation concept, which is believed to improve the quality of peppers in the global market (Chen *et al.*, 2018).

MATERIALS & METHODS

The methods described in the following subsections were improvised based on the findings from preliminary GC-MS and ^{1}H-NMR metabolomics of *P. nigrum* 'Kuching', 'Semongok Emas', 'Semongok Aman', 'India', and 'Yong Petai' fruits, collected from a pepper farm in Sebauh, Bintulu, Sarawak, Malaysia on 10th October 2018.

Collection of Plant Materials

Fresh fruit spikes bearing healthy mature fruits of the selected *P. nigrum* cultivars were collected from 13-year-old vines (as of 2023) on the same pepper farm on 25th October 2019 between 9:00 and 11:00 a.m. (Figure S1). The fruits were at developmental stage eight (8), i.e., early hard dough stage (i.e., firm, difficult to press open, and all parts of the exocarp were dark green) (Fong & Liang, 2011). The vines were planted by the farm owner on one of the farm plots. The different cultivars were identified by the owner, based on the morphological features of the leaves (Figure S2) and the fruit spikes (Figure S3).

Except for 'Kuching', the fruits spikes of eight vines were sampled for all cultivars, with one vine representing a biological replicate. For 'Kuching', only four biological replicates were available as most vines of the cultivar were in poor condition at the time of sampling. At minimum, six healthy fruit spikes were harvested from each vine. The fruit spikes from each vine were kept in labelled zip-lock bags in

an ice box and transported to a laboratory in UPM Bintulu.

In the laboratory, three fruit spikes bearing green fruits of uniform size were selected for each vine. The fruit spikes were cleaned with tap water to remove dirt and insects and dried for one hour at room temperature on unbleached parchment paper (Figure S4). To facilitate subsequent handling of the fruit spikes, the three fruit spikes representing each vine were carefully tied together with a metal wire (Figure S5). To minimise PPO activity, the bundled fruit spikes were frozen overnight at −80 °C, quenched in liquid nitrogen, and refrozen at −80 °C for 48 hours. All bundled fruit spikes were lyophilised for 48 hours and transported via air to another laboratory at the Institute of Bioscience, UPM Serdang. Then, 30 lyophilised green fruits of similar size were selected from each bundle to represent a sample (Figure S6). A dummy sample (sample code B4) was obtained from a 'Semongok Emas' sample, which was used for optimisation of GC-MS parameters and determination of representative fruit parts based on GC-MS metabolite profiles.

To further minimise fruit oxidation and facilitate their handling, fruits were stored in 15 mL polypropylene centrifuge tubes (Figure S7) in a desiccator and protected from sunlight. All samples were randomised by an individual unrelated to the study, with each sample assigned unique codes known only to the individual. Hence, the experimenters were blinded, i.e., they did not know which sample was labelled with a particular sample code, from the preparation of the extracts until the completion of the analytical procedures of the samples (Beger *et al.*, 2019; Evans *et al.*, 2020; Phapale *et al.*, 2020). The lyophilisation was carried out prior to grinding of the fruits because, as observed in the preliminary study, quenching the fresh fruits in liquid nitrogen followed by grinding them manually using mortar and pestle resulted in browning (oxidation) of the green fruits. Other than that, unlike the flat leaves, the fresh fruits were round, thus requiring more grinding time. A longer grinding time may lead to greater degree of oxidation, which can vary between the samples as the grinding was done manually. To reduce the grinding time, the lyophilised fruits were ground with a mortar and pestle, instead of the fresh fruits for each sample. Besides, of the 30 fruits per sample, only 10 fruits were ground to powder.

GC-MS Metabolomics of *P. nigrum* Fruit Extracts

GC-MS parameters in the current research were modified from previous studies (Gul *et al.*, 2017; Matsuo *et al.*, 2017; Singh *et al.*, 2013) and improvised based on the findings of the GC-MS analysis of dummy sample B4 in the preliminary study (Figures S8−S11).

Acquisition of GC-MS Data

First, 10 mg of the ground samples were weighed, extracted using 1 mL LC grade *n*-hexane containing 5 ppm *n*-nonane as an internal standard, followed by 15 min of sonication and filtration with 0.22 µm PTFE syringe filters into 1.5 mL screw-cap vials. The GC-MS system used was the 7890A Gas Chromatograph equipped with 7000 Series Triple Quadrupole Mass Spectrometer operated in electron ionisation (EI) mode at 70 eV and an ion source temperature of 200 °C. The following parameters were set for data acquisition: mode = full scan, scan rate = 10 scans/s, mass range = 30–350 Da, and solvent delay = 5 min. The inlet temperature was set at 250 °C. Then, 1 µL of extract was injected in splitless mode into a HP-5MS column of 30.00 m length × 0.25 mm internal diameter × 0.25 µm film thickness. The helium flow rate was maintained at 1 mL/min. The oven temperature was programmed as follows: 50 °C (1.0 min) and 4 °C/min to 280 °C (3.0 min), making a total run time of 61.50 min. The post-run column temperature was set at 310 °C for 1 min.

The GC-MS metabolomics workflow was adapted from a previous study, describing a four-step strategy for metabolite identification of unknown GC-MS peaks (Matsuo *et al.*, 2017). Next, noisy chromatographic peaks were excluded from further analysis by generating a calibration curve from dilution series of a pooled QC sample (sample code QCP0). For the preparation of calibration curve's QC samples (sample codes QCC1–QCC5), 5 mg of each sample (n = 36) was added to a 15 mL centrifuge tube, shaken vigorously, and the resulting mixture QCP0 (total weight: 180 mg) was divided into five 2 mL centrifuge tubes such that each tube contained 2.5 (QCC1), 3.75 (QCC2),

5.0 (QCC3), 7.5 (QCC4), and 10.0 mg (QCC5) of QCP0, respectively. The remaining QCP0 was divided into six analytical QC samples (sample codes QCP1–QCP6, 10 mg QCP0 each), which were injected after the injection of five or six samples. All QC samples (i.e., QCC1–QCC5 and QCP1–QCP6) were extracted using 1 mL LC grade *n*-hexane containing 5 ppm *n*-nonane. Three tubes containing extraction solvent blank (LC grade *n*-hexane containing 5 ppm *n*-nonane) were also prepared, labelled NH0 (not sonicated), and NH1 and NH2 (sonicated together with the samples) to monitor the background signals from the GC-MS instrument and to assess the suitability of using the signal from the internal standard to normalise the data set in PCA (Figure S12). For calculation of the LRI and the examination of the retention time shift, the alkane standard (code ALK) was injected after the first two injections of the extraction solvent blank (NH0 and NH1) and at the end of the run sequence (after the NH2 injection) (Jumhawan *et al.*, 2013). The calibration curve QC samples (QCC1–QCC5) were injected after the first injection of ALK, followed by the injection of *P. nigrum* fruit extracts and QCP1–QCP6.

Preprocessing and Principal Components Analysis (PCA) of GC-MS Data

Metabolite identification (Adams, 2017; Andriamaharavo, 2014; Babushok *et al.*, 2011; Miyazaki *et al.*, 2011), data preprocessing, and PCA followed the procedures described in a previous publication (Osman *et al.*, 2021), with modifications of several parameters in MS-DIAL version 4.60 as follows: retention time begin = min 5.48, retention time end = min 60.25, mass range begin = 40 Da, mass range end = 345 Da, number of threads = 25, amplitude cut off = 100, alignment reference file = QCC5, and normalisation = internal standard. In contrast to a previous study in which the coefficient of determination (r^2) was used to remove noisy chromatographic features (Matsuo *et al.*, 2017), the bivariate Pearson correlation coefficient (r) was used in the present research as r is more informative in modelling a linearly increasing or decreasing trend in feature intensities. A cut-off value of 0.700 was set for r when filtering the QC calibration curve (Figure S13).

^{1}H-NMR Metabolomics of *P. nigrum* Fruit Extracts

In the preliminary study, following the extraction protocol described in (Osman *et al.*, 2021), it was discovered that the aqueous methanolic fruit extracts of *P. nigrum* were unstable approximately six hours after preparation. This was evident from the formation of dark precipitates at the bottom of the NMR tubes (see Figure S14). Therefore, the sequence of steps for preparing the extracts and acquiring spectral data for 1H NMR metabolomics in the present study was designed to complete the steps involved in less than six hours. To achieve this, the procedures were carried out by two experimenters A and B in two batches of 18 extracts for each batch. All extracts (n = 36) were prepared by experimenter A and all spectral data were acquired by experimenter B. The extracts preparation for the second batch was started by experimenter A at the same time that spectral data for the extracts for the first batch were acquired by experimenter B. ^{1}H-NMR spectra were acquired, preprocessed, and analysed following the protocols detailed in the previous publication (Osman *et al.*, 2021).

LC-MS/MS Analysis

LC-MS/MS data acquisition was performed following the method described in (Osman *et al.*, 2022). The Feature-Based Molecular Network (FBMN) workflow was employed to visualise unique features in the fruit metabolite profiles of *P. nigrum* cultivars. Compared to the classical MN, which uses the summed precursor ion count or spectral count, FBMN provides more accurate quantification of relative ion intensities because FBMN uses LC-MS feature abundance such as peak area or peak height (Nothias *et al.*, 2020). To generate a feature list file for FBMN, the converted raw data were first preprocessed with MZmine 2.53 using the settings listed in Table S1. The data preprocessing settings were adjusted to match the instrument settings based on previous studies (Afzan *et al.*, 2019; Houriet *et al.*, 2020). Spectral library searches using the GNPS platform were performed following the procedure described in (Osman *et al.*, 2022). The FBMN and GNPS job parameters are publicly accessible at http://bit.ly/3ZINOFE (sample codes for the online FBMN are as follows: 01B = 'Semongok Emas', 03L = 'Semongok Aman',

03Z = 'India', 18P = 'Kuching', and 27J = 'Yong Petai').

RESULTS & DISCUSSION

GC-MS Fruit Metabolite Profiles of *P. nigrum* Cultivars

The BPCs of all 36 fruit samples of *P. nigrum* cultivars are depicted in Figure 1. All cultivars revealed similar chromatographic patterns over a run time of 61.5 min. In addition, the chromatographic peak intensities of monoterpenoids (**1**–**3**), sesquiterpenoids (**4**–**7**), and alkamides (**8**–**10**) exhibited small to moderate variations. A complete list of identified metabolites and magnified view of metabolite peaks are shown in Table S2 and Figure S15, respectively. As only representative samples are shown, the peak intensities in Figure 1 could be unique to a particular sample and do not reflect the distribution of peak intensities for all samples of the selected *P. nigrum* cultivars. Therefore, PCA was carried out to identify discriminating metabolites while accounting the distribution of peak intensities across all *P. nigrum* fruit samples.

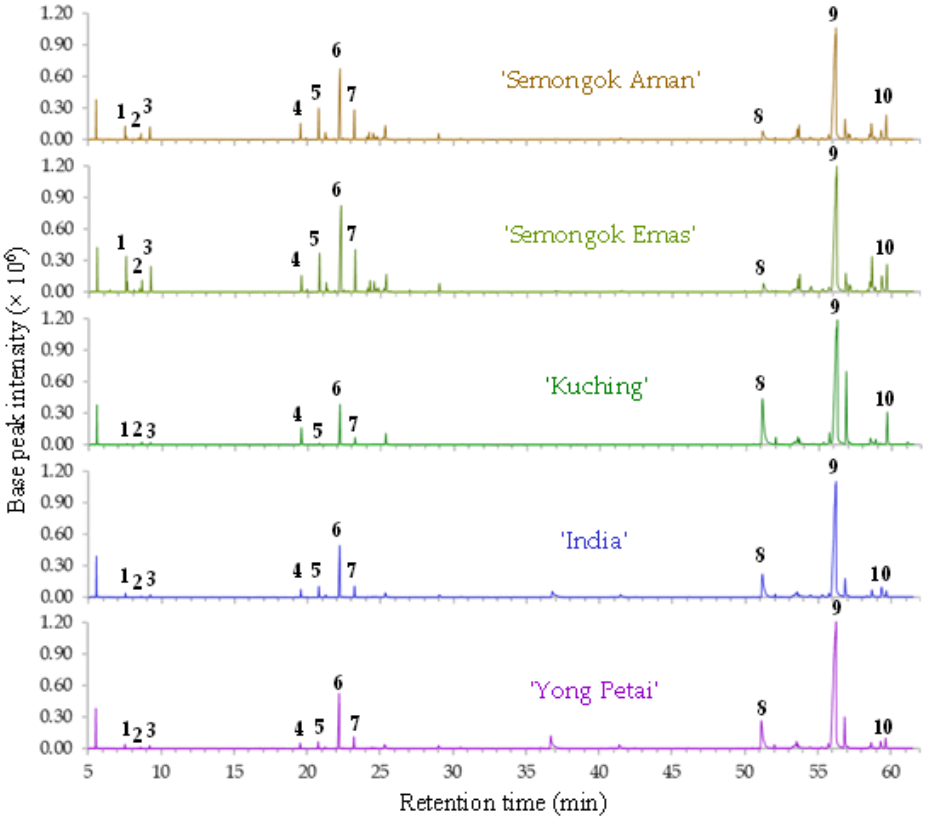

Figure 1. Representative GC-MS BPC of *n*-hexane fruit extracts of the selected *Piper nigrum* cultivars: (1) sabinene, (2) 3-carene, (3) D-limonene, (4) δ-elemene, (5) α-copaene, (6) (*E*)-β-caryophyllene, (7) α-humulene, (8) piperanine, (9) piperine, and (10) piperolein B

PCA of GC-MS Data of *P. nigrum* Fruit Extracts

Preprocessing of the GC-MS BPCs using QC samples calibration curve with r = 0.700, followed by exclusion of variables with a relative standard deviation (RSD) greater than 30% in all six QC samples, reduced the number of variables in the data set from 557 to 370. As shown in Figure 2A, with 66.6% of the total variation explained by the PCA model (see also Figure S16), the samples could be divided into three clusters: (A) a cluster of five 'Semongok Aman' samples; (B) a cluster comprising all 'Kuching' and 'Yong Petai', two 'Semongok Aman', and four 'India' samples; and (C) another cluster consisting of all 'Semongok Emas', four 'India', and one 'Semongok Aman' samples. The higher abundance of sesquiterpenoids α-copaene, β-cubebene, β-elemene, α-gurjunene,

(*E*)-β-caryophyllene, and α-humulene strongly contributes to the segregation of all 'Semongok Emas' samples (cluster C) from most other samples along the first PC, accounting 47.2% of the total variation. In addition, the lower abundance of the alkamide piperanine in the 'Semongok Emas' samples discriminates them from samples in the other two clusters. The second PC, which explains 19.4% of the total variation in the data set, highlights the significantly higher abundance of two sesquiterpenoids: germacrene B and γ-elemene (Figures 2Bviii and 2Bix) in five 'Semongok Aman' samples (cluster A) in comparison to all other samples.

Moreover, Figure 2A also shows that 'Kuching', 'Semongok Emas' and 'Semongok Aman', although they are three varieties recommended for pepper cultivation in Malaysia, have remarkable quantitative variations in their fruit metabolite profiles and are therefore not clustered in the first two PCs. Disregarding the three 'Semongok Aman' samples that deviate from the 'Semongok Aman' cluster, the clustering patterns in the PCA biplot in Figure 2A coincide well with the origins of the selected cultivars; historically, how the cultivars originated. To further highlight, *P. nigrum* is reported to have originated in the tropical forests of the Western Ghats, South India (Krishnamoorthy & Parthasarathy, 2010) and was first introduced to Sarawak by the Hakka Chinese in the 1840s Fong & Liang, 2011). 'Kuching' is one of the earliest *P. nigrum* cultivars in Sarawak and was the only commercial cultivar planted by local pepper farmers prior to introduction of the non-recommended cultivar 'India' (or 'Uthirancotta') from India in 1957. 'Kuching' and 'India' are quite similar morphologically. Both cultivars have leaves with a smooth adaxial surface and bear abundant pale-yellow flowers. Nonetheless, 'India' is considered inferior to 'Kuching' due to smaller fruit size and bear insufficient fruit caused by lower proportion of hermaphroditic flower (Fong & Liang, 2011) s.

'Yong Petai', another non-recommended cultivar in the 'Kuching' cluster (cluster B), is derived from local selection and is believed to originated from seedlings of 'Kuching'. It was found that 'Yong Petai' was grown together with 'Kuching' when it was first acquired by a pepper grower in Sungai Petai, Sarikei Division, Sarawak, in 1999 (Fong & Liang, 2011). This is supported by the finding of genetic relatedness using Directed Amplification of Minisatellite-region DNA (DAMD) markers of pepper cultivar accessions maintained at Agriculture Research Centre (ARC) Semongok, where cultivars grown in the same geographic region appear to be clustered together and 'Yong Petai' (accession no. PN129) falls in the same cluster as 'Kuching' (Ho *et al.*, 2005).

Inclusion of another half of the 'India' samples in the 'Semongok Emas' cluster reveals the genotype of 'Semongok Emas', a recommended cultivar released for cultivation by pepper farmers in 1991 after 26 years of field trials and evaluations. 'Semongok Emas' is genetically related to 'India' and 'Kuching' as it is a back-cross hybrid between 'Balancotta' (i.e., one of the four cultivars introduced from India in 1957) as the female parent and 'Kuching' as the male parent (Fong & Liang, 2011). The exclusivity of the recommended cultivar 'Semongok Aman' can be related to its country of origin, Costa Rica, where accession PN106 was collected on 21st January 1992. After several years of field evaluations, the cultivar 'Semongok Aman', resulting from clonal propagation of the germplasm of accession PN106, was released to pepper farmers in 2006 (Fong & Liang, 2011). In the aforementioned study utilising the DAMD marker, accession PN106 was separated from other accessions from Sarawak, India, and Indonesia and clustered together with several accessions from Costa Rica (PN107–112) and Honduras (PN113–116) (Ho *et al.*, 2005).

PCA of ^{1}H-NMR Spectra of *P. nigrum* Fruit Extracts

To corroborate whether the same clustering patterns are replicated by aqueous methanolic fruit extracts of the selected *P. nigrum* cultivars, the ^{1}H-NMR spectra of all samples were analysed using PCA. Figure 3A shows that the ^{1}H-NMR spectra of the different *P. nigrum* cultivars are also qualitatively similar, and no cultivar has signals that are absent in other cultivars.

The scatter plot of PCA scores in Figure 3B shows that the majority samples of 'Semongok Aman' and 'Yong Petai' can be grouped together (cluster 1) and separated from the other samples

(cluster 2) by the first PC axis. In contrast to the PCA model of the GC-MS data in Figure 2A (R^2X [cum. PC 2] = 94.9% and Q^2X [cum. PC 2] = 87.8%), the first two PCs of the ^{1}H-NMR spectral data explained only 76.4% of the total variation and the PCA model proved to be weakly predictive (Q^2X [cum. PC 2] = 63.0%). Therefore, the binned ^{1}H-NMR spectral data set subjected to power two-transformed prior to mean-centring, resulting in an improved PCA model with better fit and predictive power (Figure 3B). The power two transformation resulted in intensity changes in the variables of the data set (i.e., the binned ^{1}H-NMR signals), with only slight increases in the low-intensity variables and significant increases in the high-intensity variables. Consequently, the new PCA model was built with a greater emphasis on the variation among the high-intensity signals, which proved to be beneficial as can be seen from the improvement in the goodness of fit and prediction of the model.

Inspection of the variable loadings of the improved PCA model in Figure 3C revealed that the discrimination of the two clusters by the first PC axis was influenced by a higher abundance of variables δ 1.60, 1.68, 3.60, and 6.88 in the samples of cluster 1. As these discriminating variables are 0.04 ppm-width bins of the ^{1}H-NMR spectra of the aqueous methanolic fruit extracts of the selected *P. nigrum* cultivars, their labels in Figure 3C are the midpoints of the bins. For instance, the variable δ 3.60 in Figure 3C denotes a bin that starts at δ 3.58 and ends at δ 3.62, where δ 3.60 is the midpoint (see Figure S17). Hence, the annotation of the metabolite/s eliciting the ^{1}H-NMR signals in Figure S17 considered the whole range of signals represented by the variables δ 1.60, 1.68, 3.60, and 6.88. Besides, as the samples analysed were extracts of *P. nigrum* fruits, the probability of overlapping metabolite signals in the ^{1}H-NMR spectra was also taken into consideration.

Comparison of the ^{1}H-NMR signals in Figure S17 with the reference spectra in the Chenomx Spectral Reference Library in Chenomx Profiler version 8.2, which contain mainly ^{1}H-NMR spectra of primary metabolites and a minimal number of ^{1}H-NMR spectra of specialised metabolites in biofluids, did not show good agreement, implying that the ^{1}H-NMR signals could belong to specialised metabolite/s of *P. nigrum* fruits. Therefore, the signals were annotated based on comparison with literature data, focussing on piperine. Piperine, is a major specialised metabolite that has been utilised as a chemical marker in fingerprinting techniques for quality control of *P. nigrum* fruits (Mayr *et al.*, 2021). The annotation of the discriminatory signals largely contributed by piperine can be found in Table S3. It is possible that the discriminatory signals also originated to some extent from the three geometric isomers isochavicine, isopiperine, and chavicine, formed by photoisomerisation of piperine (Hashimoto *et al.*, 1996; Kozukue *et al.*, 2007) and the piperine analogue piperanine (Gómez-Calvario & Rios, 2019) due to co-occurrence of 1,3-benzodioxole, an unsaturated aliphatic chain, and piperidine moieties in their chemical structures. Hence, the first two PCs revealed that the aqueous methanolic fruit extracts of the recommended cultivar 'Semongok Aman' and non-recommended cultivar 'Yong Petai' had higher content of piperine and/or its geometric isomers isochavicine, isopiperine, and chavicine and/or piperanine compared to the fruit extracts of the recommended cultivars 'Kuching' and 'Semongok Emas', and non-recommended cultivar 'India'.

LC-MS/MS Metabolite Profiles of *P. nigrum* Fruits

The requirement of power two transformation of the binned ^{1}H-NMR spectra and the interpretation of the resulting PCA model with respect to the unprocessed ^{1}H-NMR spectra in Figure S17 suggest that the variation between ^{1}H-NMR fingerprints of 'Semongok Aman', 'Yong Petai', and other cultivars is small. This is evidenced by the fact that a large percentage (93.5%) of the total variation between the ^{1}H-NMR fingerprints is well-governed by merely 3.0% of the data set's variables (11 out of 370). To further investigate the nature of the variation in metabolite profiles in more polar (i.e., methanol) extracts of the selected *P. nigrum* cultivars, a sample located approximately in the centre of a cultivar's distribution of PC 1 and 2 scores in Figure 3B was selected for the acquisition of metabolite profiles with positive and negative ionisation LC-MS/MS. The fruit metabolite profiles of each cultivar were examined to select representative profiles (Figure 4), which were then visualised in the form of an FBMN. Figure 4 shows that more metabolites in methanolic fruit extracts of

selected *P. nigrum* cultivars were ionised in positive ionisation mode compared to the negative mode. While the maximum intensity of the base peak reached 12.8×10^9 ('Semongok Aman') in positive ionisation, the maximum intensity of the base peak was much lower at 1.27×10^9 ('India') in negative ionisation.

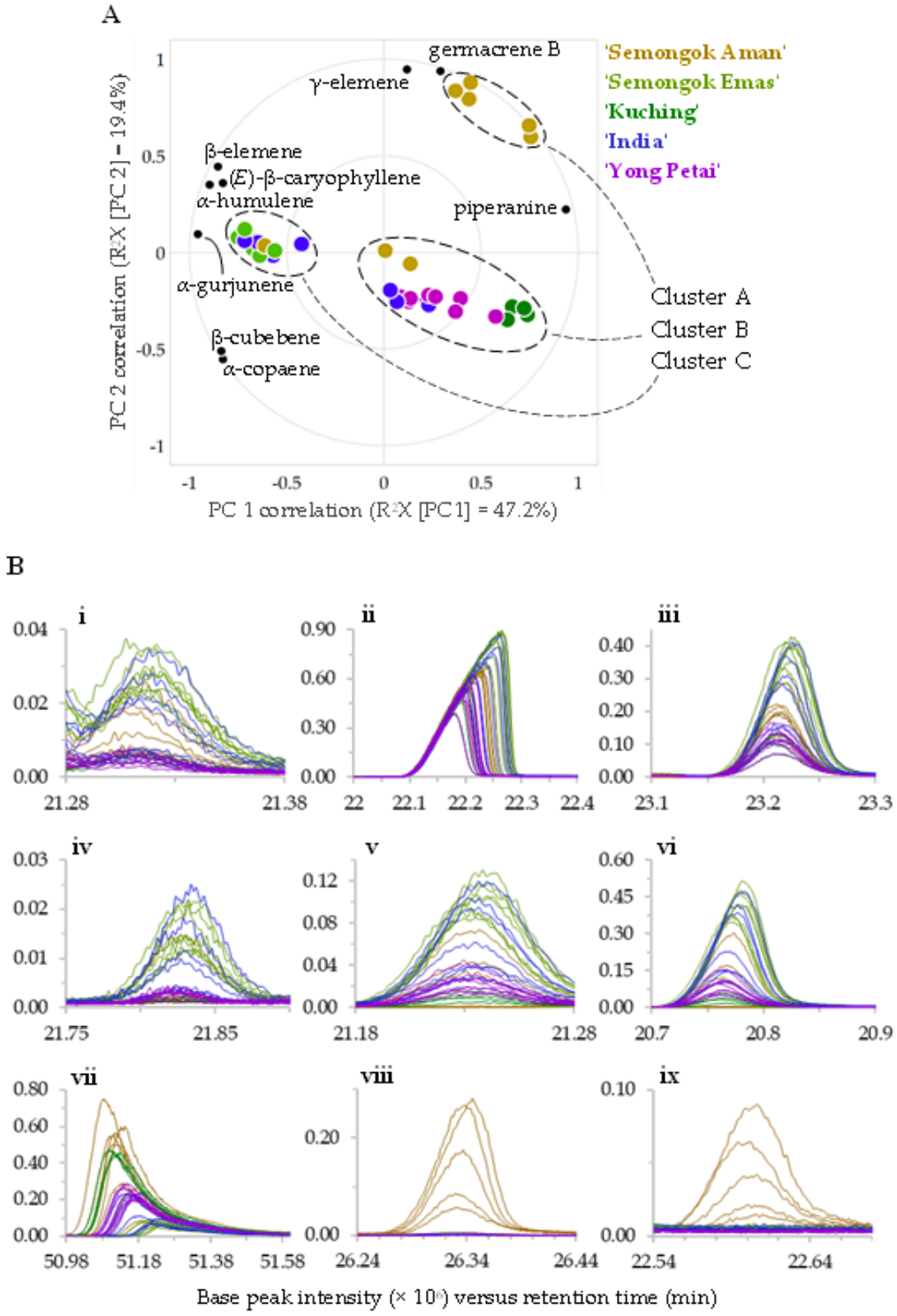

Figure 2. (A) Correlation-scaled PCA biplot of preprocessed GC-MS BPCs of *n*-hexane fruit extracts of *Piper nigrum* cultivars (Q^2X [cum. PC 2] = 61.7%) and (B) magnified views of discriminant metabolite peaks. Only well-modelled variables are shown on the biplot (Q^2VX [cum. PC 2] ≥ 80%). Dimension of data matrix = 36 × 370. (i) β-Elemene, (ii) (*E*)-β-caryophyllene, (iii) α-humulene, (iv) α-gurjunene (v) β-cubebene, (vi) α-copaene, (vii) piperanine, (viii) germacrene B, and (ix) γ-elemene

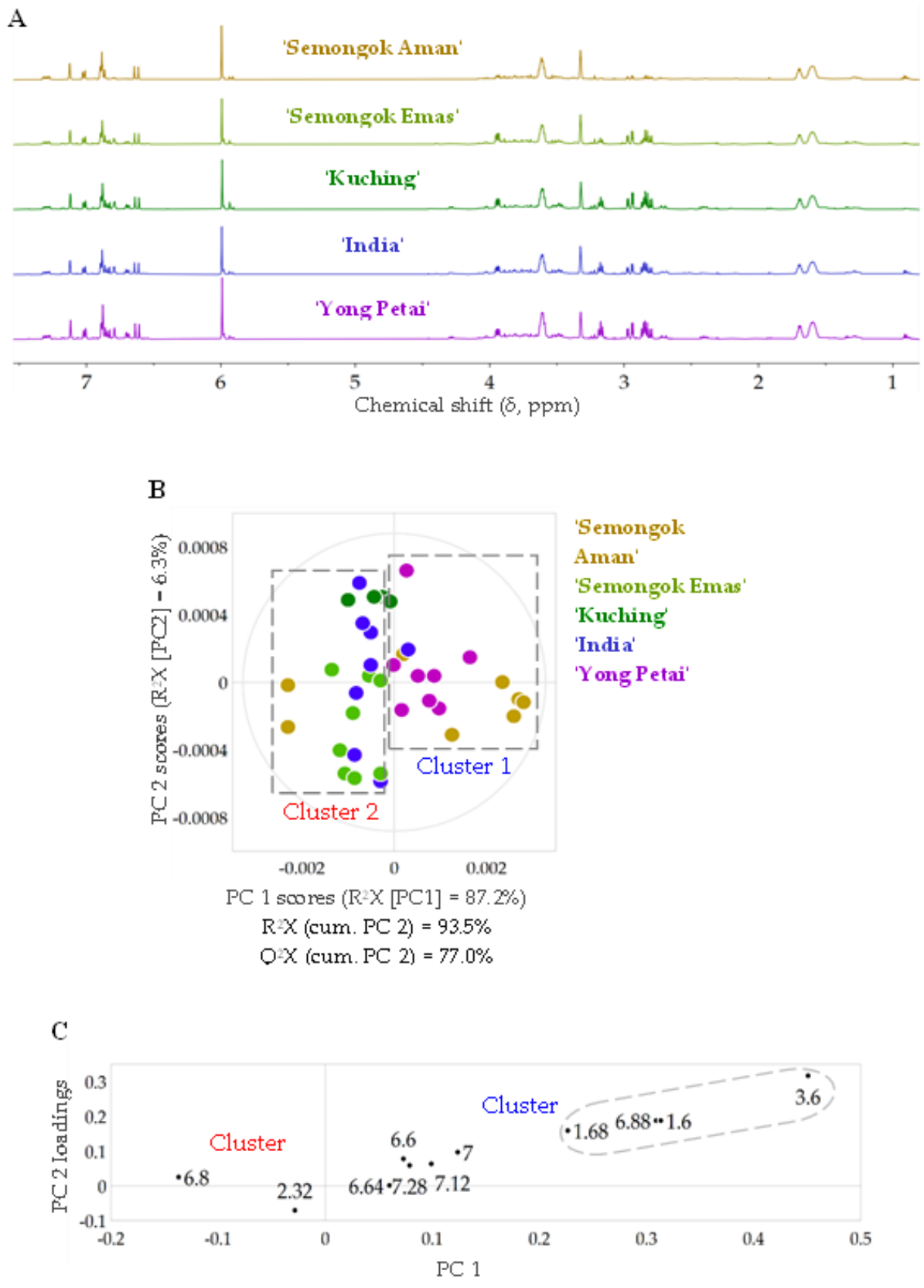

Figure 3. (A) Representative ^{1}H-NMR spectra of aqueous methanolic fruit extracts of *Piper nigrum* cultivars, (B) PCA scores scatter plot (B), and (C) loadings scatter plot of binned, power two-transformed, and mean-centred ^{1}H-NMR spectral data matrix. Dimension of data matrix = 36 × 211. All spectra in (A) were normalised to the TSP signal (δ 0.00, intensity = 100), with a maximum signal intensity = 67 (δ 6.00, 'Yong Petai'). Only well-modelled variables (Q^2VX [cum. PC 2] ≥ 80%) are shown in (C). A magnified view of the ^{1}H-NMR signals corresponding to the four high-magnitude variables circled in (C) is depicted in Figure S17

Moreover, the reproducibility of the retention time in the positive ionisation mode was found to be better than that in the negative ionisation, as the retention time shift for the base peaks at min 7.81 and by min 20.0 in the BPCs with negative ionisation can be seen in Figure 4. For these reasons, the BPCs with positive ionisation were considered to be more representative of the metabolite profiles of *P. nigrum* fruit than the BPCs with negative ionisation and were therefore selected for further examination of intercultivar metabolite profiles variation via FBMN (Figure 5 and Table 1).

Clusters A and B in the FBMN in Figure 5 were annotated as belonging to the alkamides of the *P. nigrum* fruit, whereas cluster C was annotated as belonging to the bisabolane sesquiterpene α-bisabolol. Based on GNPS spectral libraries search, the feature with the highest intensity in the positively ionised BPCs was annotated as a protonated adduct of the piperidine alkamide piperine (cluster B, node ID 1). Therefore, the higher base peak intensities of protonated piperine in 'Semongok Aman' (12.8×10^9) and 'Yong Petai' (12.5×10^9) relative to the other cultivars ($11.4–12.0 \times 10^9$) in Figure 4 support the grouping of the two cultivars into cluster 1 in ^{1}H-NMR metabolomics. Nevertheless, the ID 1 node in Figure 5 implies that the intensity of the base peaks of protonated piperine for each cultivar differs only slightly. This is parallel with the findings described previously, i.e., the contribution of piperine signals to the intercultivar metabolite profile variation is minimal. Apart from piperine, the intensity of the base peaks of other specialised metabolites is also similar in each cultivar, with the exception of node IDs 226 and 4 of cluster A, node 21 of cluster B and node 1285 of cluster C. These four FBMN nodes have significantly higher base peak intensities of the piperidine alkamide piperanine, two isobutylamine alkamides, namely (*E*)-5-(1,3-benzodioxol-5-yl)-*N*-(2-methylpropyl)pent-4-enamide and piperlonguminine, and the sesquiterpene α-bisabolol in the recommended cultivar 'Semongok Aman'.

The experimental and data analysis workflow in this exploratory research enabled the detection of fruit metabolite profiles and fingerprint patterns of the recommended and non-recommended Malaysian *P. nigrum* cultivars via utilisation of GC-MS and ^{1}H-NMR, respectively. In addition, FBMN of the fruit's LC-MS/MS data was employed to carry out automated metabolite annotation, focusing on the metabolites that led to the discriminating ^{1}H-NMR signals.

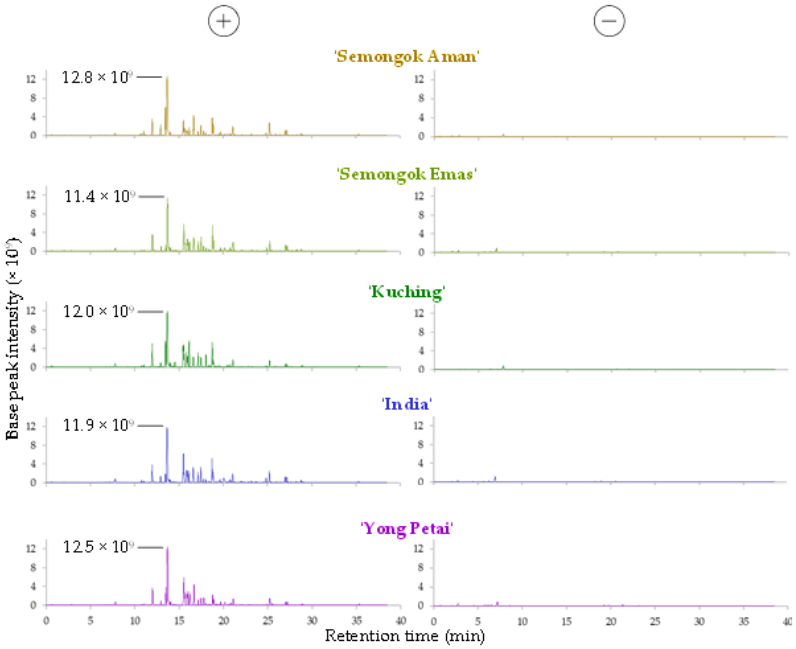

Figure 4. Positive (left) and negative (right) ionisation LC-MS BPCs of methanolic fruit extracts of *Piper nigrum* cultivars

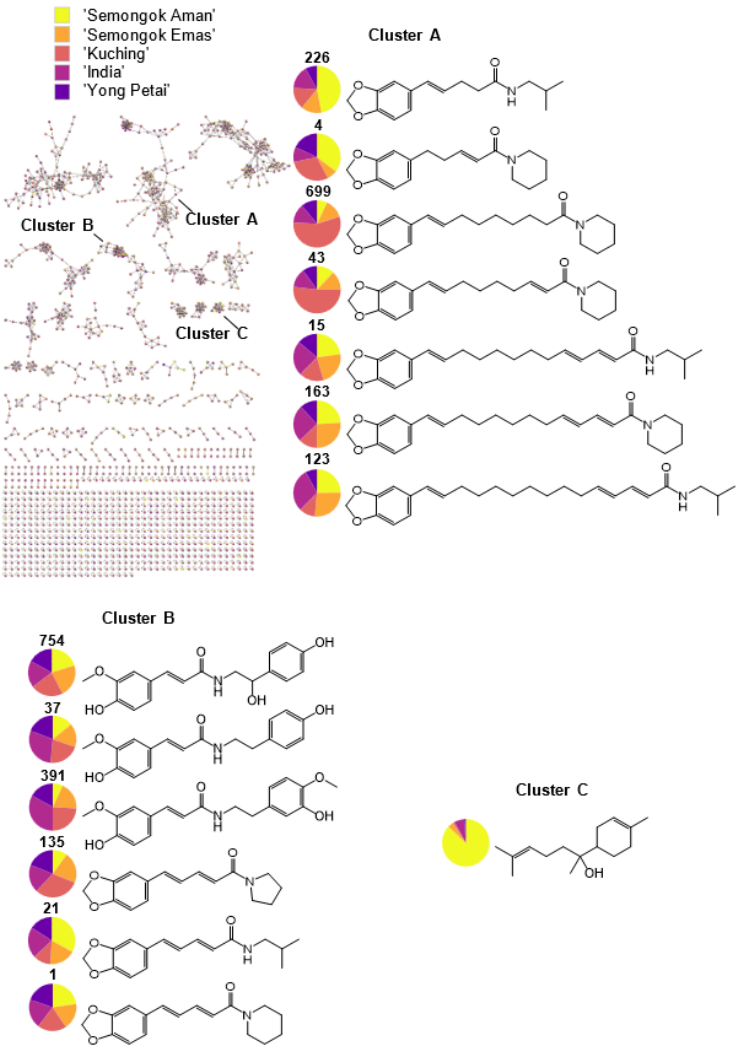

Figure 5. Positive ionisation BPCs FBMN of methanolic fruit extracts of *Piper nigrum* cultivars. The numbers above the FBMN nodes are the node IDs. The FBMN nodes indicate the percent intensity of precursor ions in all samples. See Table 1 for the description of the metabolites

In this research, GC-MS was preferred over ^{1}H-NMR spectroscopy because, apart from the analytical challenge due to the lack of ^{1}H-NMR reference spectra of specialised metabolites in the Chenomx spectral library and the computational challenge of deconvoluting the ^{1}H-NMR fingerprint, to annotate metabolites in complex mixtures (Häckl *et al.*, 2021; Mehr *et al.*, 2023; Schmid *et al.*, 2023), the annotation of discriminative metabolite signals in the fingerprint generated from a small sample size (30 mg) is even more challenging when the percentage of variation to be examined (i.e., infraspecific variation) is much smaller than the interspecific variation. Moreover, previous studies have pointed out the issue of low solubility of piperine in methanol and water (Traxler *et al.*, 2020) and the potential problem of locking and shimming during NMR spectra acquisition due to the use of mixed solvents (Rivera-Pérez *et al.*, 2022). Nevertheless, considering the rapid acquisition of ^{1}H-NMR spectra (whose file sizes are much smaller than those of LC-MS/MS), which precludes time-consuming optimisation of instrument settings and multiple steps of data preprocessing as in LC-MS metabolomics. It is important to highlight that the ^{1}H-NMR metabolomics allowed further overview of clustering of the *P. nigrum* cultivars apart from GC-MS metabolomics. While the same clustering pattern was recognised in both GC-MS and ^{1}H-NMR metabolomics, the inclusion of chromatography and *m/z* dimensions in the GC-MS and LC-MS/MS datasets facilitated the annotation of the discriminating metabolites underlying the observed clustering patterns.

Table 1. List of metabolites identified via GNPS spectral library searches in the FBMN workflow

Average RT (min)	Node ID	Metabolite	Molecular formula	Adduct	*m/z*	Chemical class
Cluster A						
12.87	226	(*E*)-5-(1,3-benzodioxol-5-yl)-*N*-(2-methylpropyl)pent-4-enamide	$C_{16}H_{21}NO_3$	$[M+H]^+$	276.159	Isobutylamine alkamide
13.50	4	Piperanine	$C_{17}H_{21}NO_3$	$[M+H]^+$	288.159	Piperidine alkamide
18.06	699	Piperolein B	$C_{21}H_{29}NO_3$	$[M+H]^+$	344.223	Piperidine alkamide
18.07	43	Pipernonaline	$C_{21}H_{27}NO_3$	$[M+H]^+$	342.206	Piperidine alkamide
21.10	15	Guineensine	$C_{24}H_{33}NO_3$	$[M+H]^+$	384.253	Isobutylamine alkamide
22.16	163	Piperchabamide C	$C_{25}H_{33}NO_3$	$[M+H]^+$	396.253	Piperidine alkamide
23.21	123	Brachystamide B	$C_{26}H_{37}NO_3$	$[M+H]^+$	412.285	Isobutylamine alkamide
Cluster B						
6.87	754	(*E*)-*N*-[2-hydroxy-2-(4-hydroxyphenyl)ethyl]-3-(4-hydroxy-3-methoxyphenyl)prop-2-enamide	$C_{18}H_{19}NO_5$	$[M+H]^+$	330.133	Tyramine alkamide
7.81	37	Moupinamide	$C_{18}H_{19}NO_4$	$[M+H]^+$	314.139	Tyramine alkamide
8.12	391	(*E*)-3-(4-hydroxy-3-methoxyphenyl)-*N*-(3-hydroxy-4-methoxyphenethyl)acrylamide	$C_{19}H_{21}NO_5$	$[M+H]^+$	344.149	Tyramine alkamide
12.30	135	Trichostachine	$C_{16}H_{17}NO_3$	$[M+H]^+$	272.120	Pyrrolidine alkamide
12.96	21	Piperlonguminine	$C_{16}H_{19}NO_3$	$[M+H]^+$	274.144	Isobutylamine alkamide
13.69	1	Piperine	$C_{17}H_{19}NO_3$	$[M+H]^+$	286.144	Piperidine alkamide
Cluster C						
19.93	1285	α-Bisabolol	$C_{15}H_{26}O$	$[M+H-H_2O]^+$	205.195	Bisabolane sesquiterpene

Although three of the eight 'Semongok Aman' samples exhibited individual variations that led to their segregation from the main cluster, the fruits of 'Semongok Aman' originating from Costa Rica were generally found to have a more distinct metabolite profile and fingerprint than the four other, more genetically similar cultivars. The difference was evident in two metabolite classes, namely sesquiterpenoids (germacrene B, γ-elemene, and α-bisabolol) and piperidine alkamides (piperanine, piperine, and piperine isomers). Apart from having better tolerance than 'Kuching' to Phytophthora foot rot caused by the oomycete *Phytophthora capsici* and blackberry diseases caused by the fungal plant pathogens *Colletotrichum truncatum*, *gloeosporioides*, and *orchidearum* species complexes (Jayawardena *et al.*, 2021), and infestation by the pepper stem borer (*Lophobaris piperis*), 'Semongok Aman' is considered a cultivar with satisfactory fruit yield, uniform fruit ripening, and good chemical quality (Fong & Liang, 2011). However, 'Semongok Aman' and 'Kuching' are equally susceptible to the major disease known as slow decay/wilt. This is a debilitating disease complex between the root-knot nematode *Meloidogyne* spp. and the plant-pathogenic fungus *Fusarium* spp. (Fong & Liang, 2011). In one study, the significant increase in sesquiterpenoids α-bisabolol and δ-elemene in the leaves of 'Bragantina' and 'Cingapura', respectively (two important *P. nigrum* cultivars in Brazil), observed after infection with *Fusarium solani* f.

sp. piperis, was postulated to be a response to plant-pathogen interaction (Trindade *et al.*, 2021). Therefore, from the viewpoint of biotechnological approach for the improvement of *P. nigrum* cultivars, future research focusing on the biosynthetic pathways of sesquiterpenes in existing *P. nigrum* cultivars with a significantly higher content of sesquiterpenoids content may assist in the identification of candidate genes for resistance to *Fusarium* spp.

CONCLUSION

The current research provided further information regarding the unique chemical quality of 'Semongok Aman', which was characterised by a higher abundance of the sesquiterpenoids germacrene B, γ-elemene, and α-bisabolol, and the piperidine alkamides piperanine, piperine, and piperine isomers than the other four Malaysian *P. nigrum* cultivars. The findings are parallel with the current recommendation of the Malaysian Pepper Board that 'Semongok Aman' should be grown in local pepper farms over the other non-recommended cultivars due to its good disease tolerance profile, superior chemical quality, and better annual fruit yield and fruit ripening uniformity. In terms of monovarietal pepper cultivation to boost Malaysian pepper production in the world market, 'Semongok Aman' could be considered as a suitable cultivar for future implementation of such an approach.

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DATA AVAILABILITY

Metabolomics data have been deposited in the EMBL-EBI MetaboLights database with identifier number MTBLS7768 (https://www.ebi.ac.uk/metabolights/editor/study/MTBLS7768/descriptors).

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