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Molecular Characterization and Metabolite Profiling of Philippine *Allium sativum* Linn.: Ilocos Pink

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Ilocos Pink garlic (IPG) is a local garlic variety found in Ilocos Norte, Philippines. Recently known for its moderate beta-adrenergic receptor inhibitory activity *in vivo*, there is still a limited number of studies describing its genetic and metabolite profile to distinguish it from other garlic varieties. In this study, genetic markers of IPG were identified using sequence-related amplified polymorphism (SRAP) analysis. Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry followed by principal component analysis (PCA) was used to discriminate IPG's metabolites from Ilocos Native garlic. Based on the degree of brown-stripe pigmentation on their outer skin, IPG samples can be classified into three – light, moderate, and heavy pigmentation. These subgroups were found to share seven SRAP marker pairs – namely, ME1-EM1 (at 300bp), ME1-EM4 (at 400bp), ME2-EM3 (500bp), ME3-EM1 (300bp), ME3-EM2 (at 400bp), ME3-EM4 (at 200bp), and ME5-EM2 (at 300bp). Unique SRAP marker pairs were also observed between subgroups. PCA revealed Ilocos Native garlic to be discriminated from the IPG groups, but the marker matrix tool showed mere differences in concentrations except m/z 247.129 at RT 1.40. Concentration-wise, nine markers may be proposed to discriminate IPG light from IPG moderate and heavy, seven of which are putatively identified as saponins. These findings suggest that SRAP markers can effectively discriminate IPG into subgroups, whereas metabolite profiling may provide little insight into the differences between IPG and Ilocos Native garlic.

Keywords: *Allium sativum*, Ilocos Pink garlic, mass spectrometry, principal component analysis, sequence-related amplified polymorphism

INTRODUCTION

Characterization and inter- and intra-species discrimination are important in medicine and plant biology to identify specific morphological and biochemical traits, as well as their changes related to stress (Clauw *et al.* 2015), provide molecular information for germline selection and crop breeding (Govindaraj *et al.* 2015), measure and assess the extent of tolerance against various environmental conditions (Daniels *et al.* 2015; El-Esawi and Alayafi 2019), and discover novel compounds for drug discovery (Najmi *et al.* 2022). Morphology-based methods have long been used to characterize plant species diversity. However,

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these methods have several limitations including their inability to assess diversity in the presence of insignificant heritable trait variations, limited numbers of traits to characterize, and high trait variability influenced by plant maturity, environmental conditions, and cultivation systems (Blazakis *et al.* 2017).

Metabolite profiling is also being considered an approach to fully characterize plant species and cultivars. Unlike morpho-anatomical analysis, it is used to delineate species diversity through the examination of their regulatory and physiological mechanisms, as reflected by the identity and amount of their metabolites (Salem *et al.* 2020). It can provide information on the capacity of plants to tolerate extreme salinity conditions (Xie *et al.* 2020; Chen *et al.* 2021) and drought (You *et al.* 2019), as well as describe its nutrient composition (Rachkeeree *et al.* 2018; Kumar *et al.* 2021). In addition, this can not only supply useful information on the characterization of germplasm with respect to geographic regions (Abdelrahman *et al.* 2021) but also aid in assessing genetic diversity (Hrbek *et al.* 2018) and their potential in discovering novel compounds with medicinal properties (Bontempo *et al.* 2021). Despite these advantages, the limitations of metabolic analysis include plant metabolite complexity, expensive cost, incomplete knowledge of plant metabolic pathways, and additional analytical techniques to further characterize the broad range of metabolomic findings (Tugizimana *et al.* 2013). Thus, other approaches are necessary to fully support the characterization of plant species and cultivars.

DNA-based systems have been an invaluable tool for assessing plant species diversity, as they supplement the morpho-anatomical and biochemical characterization of plants. Several DNA-based methods include random amplified polymorphic DNA (Singh *et al*. 2014), amplified-fragment length polymorphisms (AFLP) (Egea *et al*. 2017), simple-sequence repeats (SSR) (Kumar *et al.* 2019), insertions-deletions (Wang *et al.* 2016), and sequence-related amplified polymorphisms (SRAP) (Nadeem *et al.* 2018). Among these, SRAP was found to be more robust in presenting genetic diversity among homogeneous cultivars and germplasms (Robarts and Wolfe 2014). It is capable of detecting genetic variations in a short amount of time, making it a powerful polymerase chain reaction (PCR) based molecular system. In identifying codominance among examined species, SRAP produced a higher detection yield as compared to AFLP (Agyenim-Boateng *et al.* 2019). It has also been utilized in constructing genetic linkage maps (Guo *et al.* 2014), identifying quantitative trait loci (Robarts and Wolfe 2014), and cDNA fingerprinting and gene tagging (Wu *et al.* 2013). Furthermore, SRAP is reproducible with minimal technical effort having a simple manner of reporting (scoring of 0 or 1) and a single-step protocol for PCR (Robarts and Wolfe 2014). SRAP analysis is also cost-effective with a relatively reasonable throughput rate, especially in discriminating genetic diversity of inter- and intraspecies of plants (Li *et al.* 2013).

Garlic (*Allium sativum* Linn), locally known in the Philippines as *bawang*, is a perennial flowering bulbous plant that is commonly used in cooking and traditional medicine. Originating from Central Asia 4,000 years ago, it is now widely cultivated across regions with an array of varieties (Petrovska and Cekovska 2010). In the Philippines, seven garlic cultivars are registered in the Bureau of Plant Industry–National Seed Industry Council (BPI-NSIC) – namely, Ilocos White, Batanes Red, Ilocos Pink, Mexican, Bang-ar', Ilocos Tan Bolters, and MMSU Gem (BPI-NSIC 2021). Ilocos Pink (IPG), locally known as *gameng* in Ilocos Norte, is characterized by pink cloves and white with brown stripes outer skin. It is among the Philippine garlic variants with a unique number of days for maturity and color of skin (BPI-NSIC 2021). A previous study has demonstrated moderate beta-adrenergic receptor inhibitory activity of IPG extracts *in vivo* (Genuino 2018). SRAP was found to be a good discriminating molecular tool in assessing IPG's genetic profile compared to *rbcL* and *matK* gene primer systems (Bigtas *et al.* 2020). However, a recent IPG collection showed differences in its morphological structure, particularly the level of brown stripes in the outer skin of IPG. These phenotype variations suggest differences in their genetic profile, which may affect their bioactivities. With a limited number of studies exploring IPG's molecular diversity and metabolite profile, this study aimed to discriminate its biochemical and genetic diversity based on well-defined morphological variations.

MATERIALS AND METHODS

Acquisition of Samples, Selection, and Sorting

IPG specimens were purposively acquired from the Pasuquin, Ilocos Norte, Philippines last March 2022. Specimens were sorted according to the difference in overall quality and status (with or without damage). ImageJ, an image-processing software, was used to calculate and determine the pixel values or area of the characteristic brown stripes of the IPG outer skin. The categorized garlic samples were further divided into two groups – one for genetic analysis and one for metabolite profiling.

DNA Extraction

Five garlic cloves from the categorized garlic bulb specimens were used for DNA extraction. Garlic cloves

were peeled and half-submerged in water with 0.5 µL of indole-3-butyric acid to allow root growth. Roots were allowed to grow for 4–5 d at room temperature in a sterile condition to prevent contamination. DNA extraction was performed following the modified cetyltrimethylammonium bromide (CTAB) method adapted from Allen and colleagues (2006), which uses ethanol instead of cesium chloride as the precipitating agent. Quantitation of DNA purity was determined using DNA NanoDrop Spectrometer (Thermo Scientific).

Polymerase Chain Reaction (PCR) Amplification, Agarose Gel Electrophoresis, DNA Sequencing, and Sequence-related Amplified Polymorphism (SRAP) Analysis

Twenty-five (25) SRAP-marker pairs adapted from the work of Bigtas *et al.* (2020) were used in PCR amplification using Bio-rad T-100 Thermocycler (USA). Genomic DNA was amplified using the Promega PCR kit (USA). The PCR reaction mixture was slightly modified by increasing the magnesium chloride ($MgCl_2$) concentration to accommodate the increased primer melting temperature, thereby improving the primer-template DNA interactions. A 10-µL PCR reaction mixture of the SRAP markers was prepared and is composed of the following: 0.2 µL of 10 mM forward and reverse primers, 1 µL of DNA template (20 ng/µL), 0.2 µL of 40 mM dNTPs, 2.0 µL of GoTaq Buffer, 0.05 µL of GoTaq DNA Polymerase, 2.4 µL of $MgCl_2$, and 3.95 µL of nuclease-free water. The thermal cycle profile was set according to the manufacturer's protocol. The SRAP primer sequences and the utilized thermal profile for amplification are shown in Table 1. The PCR products were subjected to agarose gel electrophoresis using 1% agarose in 0.5x TAE buffer and 1 µL gel red dye at 110 V for 45 min. Amplified polymorphic bands were then visualized in a gel documentation system (Bio-Rad, USA).

Metabolite Profiling

Five representative garlic bulbs were obtained from the Ilocos Pink groupings and from Ilocos Native garlic, which served as the control group. Samples were manually crushed using mortar and pestle. One gram (1 g) of the sample was extracted with 2.5 mL of LCMS-grade methanol by sonication for 30 min, followed by centrifugation for 10 min at 4000 x g. Extraction was repeated thrice following the same protocol. Supernatants were pooled in a 15-mL conical tube and brought to a final volume of 10 mL. The samples were then passed through a 0.22-µm polytetrafluoroethylene polymer syringe filter prior to injection.

Metabolite analysis was carried out using Waters UPLC I-Class coupled with Xevo G2-XS qTof mass spectrometer (Waters Corp., USA). The column used was a 2.1 × 100 mm x 1.8-µm Acquity HSS T3. The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with the gradient time program of 0 → 0.5 min 95% A and then 0.5 → 15 min 5% A. The flow rate was set at 0.5 mL/min with an injection volume of 1 µL.

As for the mass spectrometry method, it follows the MS^E principle – where fragmentation is ramped from low to high energy. It uses the following parameters: MS^E small molecules screening acquisition mode, capillary voltage: 3 kV, source temperature: 120 °C, desolvation temperature: 550 °C, cone voltage: 30V, cone gas flow: 50 L/h, and desolvation gas flow: 950L/h. The analysis was conducted in three injection replicates in positive ESI with an m/z range of 50–1,200. Leucine enkephalin was used as a reference fluidic for mass correction. System suitability was performed using a quality control (QC) mix composed of equal amounts of the samples. The MS data were acquired and processed through UNIFI

Table 1. Primers that were used to amplify the *Allium sativum* Linn: Ilocos Pink SRAP regions and the corresponding thermal profile.

Marker	Primer name	Primer sequence (5' – 3')	Thermal profile
SRAP	ME1	5'- TGA GTC CAA ACC GGA TA - 3'	94 ºC for 5 min, 94 ºC for 1 min, 35 ºC for 1 min, and 72 ºC for 1 min, 5 cycles; 94 ºC for 1 min, 50 ºC for 1 min, 72 ºC for 1 min, 35 cycles, and 72 ºC for 5 min
	ME2	5'- TGA GTC CAA ACC GGA GC - 3'	
	ME3	5'- TGA GTC CAA ACC GGA AT - 3'	
	ME4	5'- TGA GTC CAA ACC GGA CC - 3'	
	ME5	5'- TGA GTC CAA ACC GGA AG - 3'	
	EM1	5'- GAC TGC GTA CGA ATT AAT - 3'	
	EM2	5'- GAC TGC GTA CGA ATT GAC - 3'	
	EM3	5'- GAC TGC GTA CGA ATT TGA - 3'	
	EM4	5'- GAC TGC GTA CGA ATT AAC - 3'	
	EM5	5'- GAC TGC GTA CGA ATT GCA - 3'	

[ME] forward primer; [EM] reverse primer

Scientific Information System ver. 1.8 software (Waters Corp., USA). The masses were screened for their putative identification using the Traditional Chinese Medicine Library (Ehrman *et al.* 2007) and an in-house library composed of reported metabolites of *Allium sativum.* The mass match has an accuracy of three significant figures (< ± 10 mDa). Only the parent compounds whose *in silico* fragmentation patterns detected in the MS^E fragmentation sample database are considered as identified with confidence.

Data Analysis

Garlic samples were categorized using Ward's method hierarchal cluster analysis by SPSS (version 27.0), followed by Tukey's *post hoc* test ($p < 0.05$). The metabolite data from the UNIFI Scientific Information System were imported to the EZInfo software (Version 2.0, Germany) for the principal component analysis (PCA) to elucidate differences in the metabolites of IPG groups. Loading plots, which highlight potential markers expressed through exact mass/retention time (EMRT) pairs, were also obtained from the correlation of the scores plot. A trending plot was used to further discriminate the potential markers in each sample group according to their relative abundance.

RESULTS

Garlic Sorting Using ImageJ

A total of 178 IPG bulbs were subjected to ImageJ analysis (Figure 1a). The ratio of the measured brown pigment area on the outer skin of the IPG and the total area of the garlic bulbs were computed and used to categorize the samples into three groups: light, moderate, and heavy (Figure 1b). The majority of the samples tested (n = 100) were classified under the IPG light group having a brown pigmentation-to-bulb size ratio of 0.4–8.9. For the moderate IPG group, only 59 out of 178 tested IPG samples were considered with a brown pigmentation-to-bulb size ratio of 9.4–22.40. Only 19 IPG samples were categorized under the heavy IPG group having a brown pigmentation-to-bulb size ratio of 24.20–40.6.

Sequence-related Amplified Polymorphism (SRAP) Analysis

Twenty (20) SRAP marker pairs were identified that can discriminate IPG and classify it into subgroups (Figure 2). In particular, seven SRAP marker pairs are shared by the three groups. The IPG light group can be described by six unique SRAP marker pairs, whereas the IPG moderate group has three unique *SRAP* markers (Table 2). Both IPG light and moderate groups displayed polymorphism at 400bp in the ME1-EM2 SRAP marker pair. The IPG heavy group, on the other hand, distinctly displayed

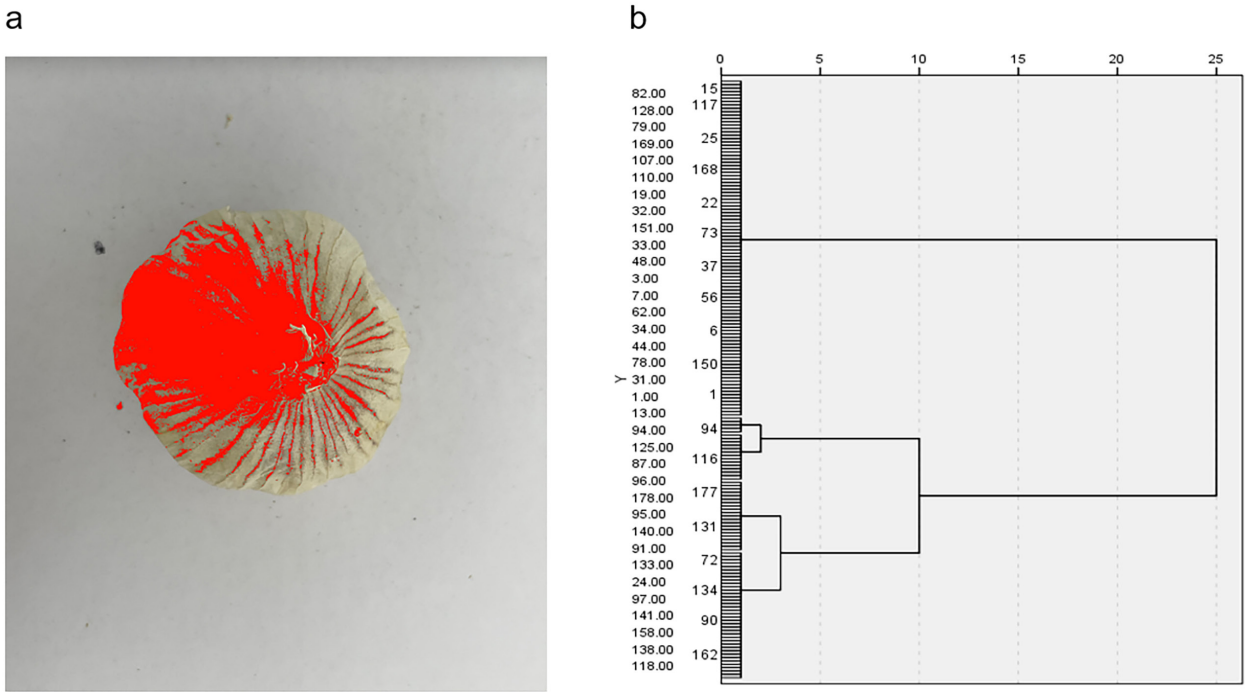

Figure 1. [a] Representative image of *Allium sativum* Linn: Ilocos Pink (IPG) sample from the heavy group subjected to Image J analysis. [b] Dendrogram of *Allium sativum* Linn: Ilocos Pink (IPG) samples from Ilocos.

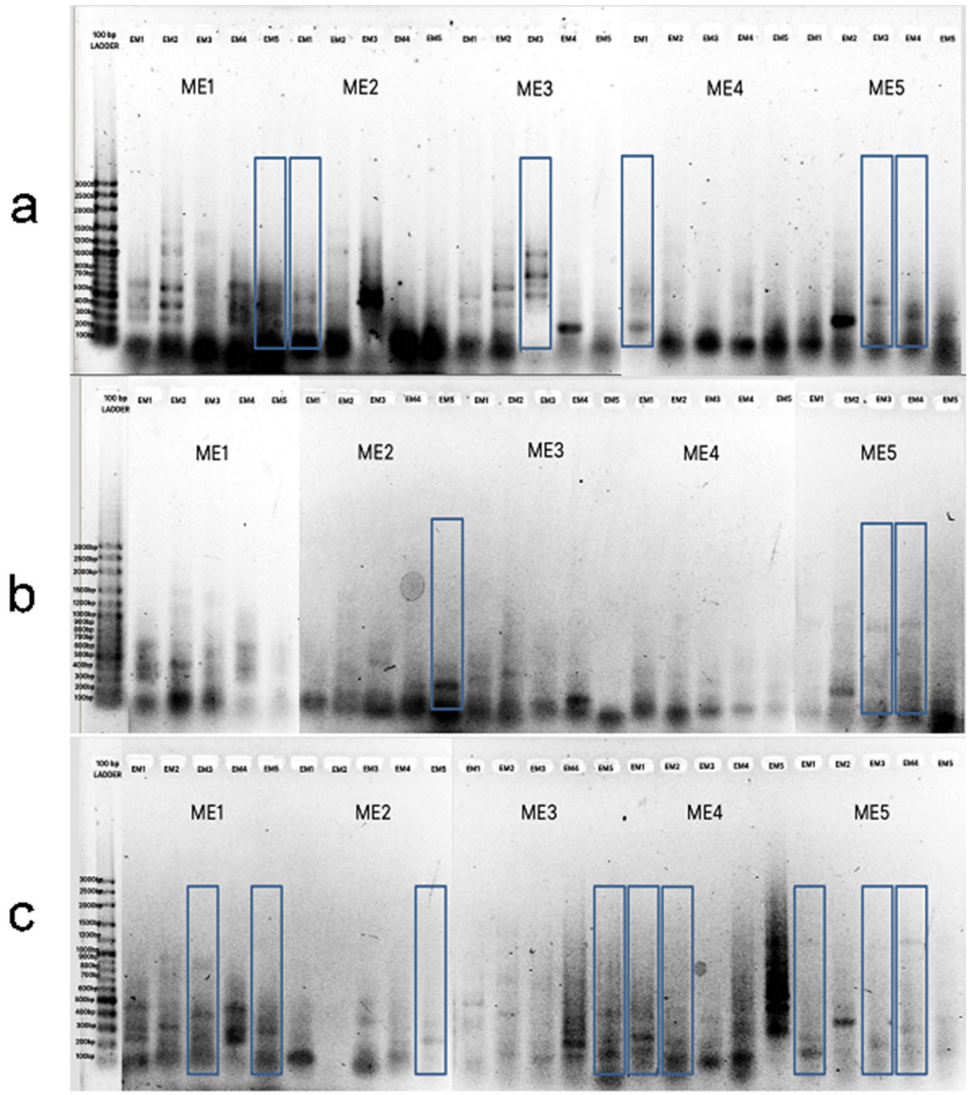

Figure 2. Gel profile showing PCR amplicons from SRAP primer combinations of the three *Allium sativum* Linn: Ilocos Pink (IPG) subgroups: light (a), moderate (b), and heavy (c) resolved in 1% agarose gel. Three groups shared seven primer pairs and unique primer pairs were also observed per group (blue boxes).

Table 2. Summary of the *Allium sativum* Linn: Ilocos Pink SRAP analysis.

Unique primers	Similar primers pairs (pairwise)			Shared primer pairs
Light				ME1-EM1 (300bp)
				ME1-EM4 (400bp)
				ME2-EM3 (500bp)
	Light	Moderate	Heavy	ME3-EM1 (300bp)
				ME3-EM2 (400bp)
				ME3-EM4 (200bp)
				ME5-EM2 (300bp)

Table 2. Continued . . .

	ME1-EM5 (600bp, 400bp) ME2-EM1 (500 bp, 300bp) ME3-EM3 (1000bp, 700bp, 500bp) ME4-EM1 (300bp) ME5-EM3 (500bp) ME5-EM4 (400bp)		ME1-EM2 (400bp)	ME1-EM2 (300bp)
Moderate	ME2-EM5 (300bp) ME5-EM3 (800bp) ME5-EM4 (900bp)	ME1-EM2 (400bp)		
Heavy	ME1-EM3 (400bp) ME1-EM5 (250bp) ME2-EM5 (200bp) ME3-EM5 (400bp 200bp, 150bp) ME4-EM1 (200bp, 150bp) ME4-EM2 (250bp 200bp) ME4-EM5 (1200bp, 900bp, 600bp, 400bp, 300bp) ME5-EM1 (200bp, 150bp) ME5-EM3 (200bp) ME5-EM4 (1200bp, 300bp)	ME1-EM2 (300bp)		

polymorphism across 10 SRAP marker pairs. Likewise, it shares SRAP marker pairs with IPG light group in ME1-EM2 (at 300bp). These results suggest that SRAP analysis can be used to distinguish the molecular uniqueness and variety of IPG and its subgroups.

Metabolite Profiling

Overlaid chromatograms of the LCMS metabolic analysis (Figure 3) showed 55 peaks common among the IPG groups and Ilocos Native (control group). A distinct peak at retention time 1.40 was found in Ilocos Native differentiating it from IPG. Among the 55 peaks, 36 were putatively identified based on traditional Chinese medicine (TCM) (Ehrman *et al.* 2007) and customized libraries (Table 3).

Eleven (11) of the putatively identified compounds were comprised mainly of amino acids and peptides based on the in-house library. The other 25 were matched to the TCM – composed of terpenoid, phenolic, and saponin compounds.

Principal Component Analysis (PCA)

PCA, in addition to overlayed chromatograms, revealed one metabolite – m/z 247.129 at RT 1.40 – that can discriminate Ilocos Native from IPG groups. Interestingly, IPG light showed a considerable distance from both IPG moderate and heavy clusters. Subsequently, loading plots were correlated to the PCA plot (Figure 4). The EMRT pairs clustered in the middle of the diagram are the secondary metabolites shared among the four groupings with similar relative abundance. The encircled EMRT pairs are the potential chemical markers of each of the four groupings – red for IPG light, green for IPG moderate, black for IPG heavy, and blue for the Ilocos Native.

The Unifi software provides a marker matrix table and the corresponding marker summary in which the abundance of the potential markers in each group is plotted. The potential chemical markers of IPG are summarized in Table 4. None among the EMRT pairs in the loadings plot showed uniqueness to a specific group only. Instead, their potentials as chemical markers are based only on relative abundance differences, wherein one group shows higher counts than the other two or *vice versa.* For instance, the nine chemical markers of IPG light are proposed as they are detected in this group at a higher intensity as compared to IPG moderate and heavy.

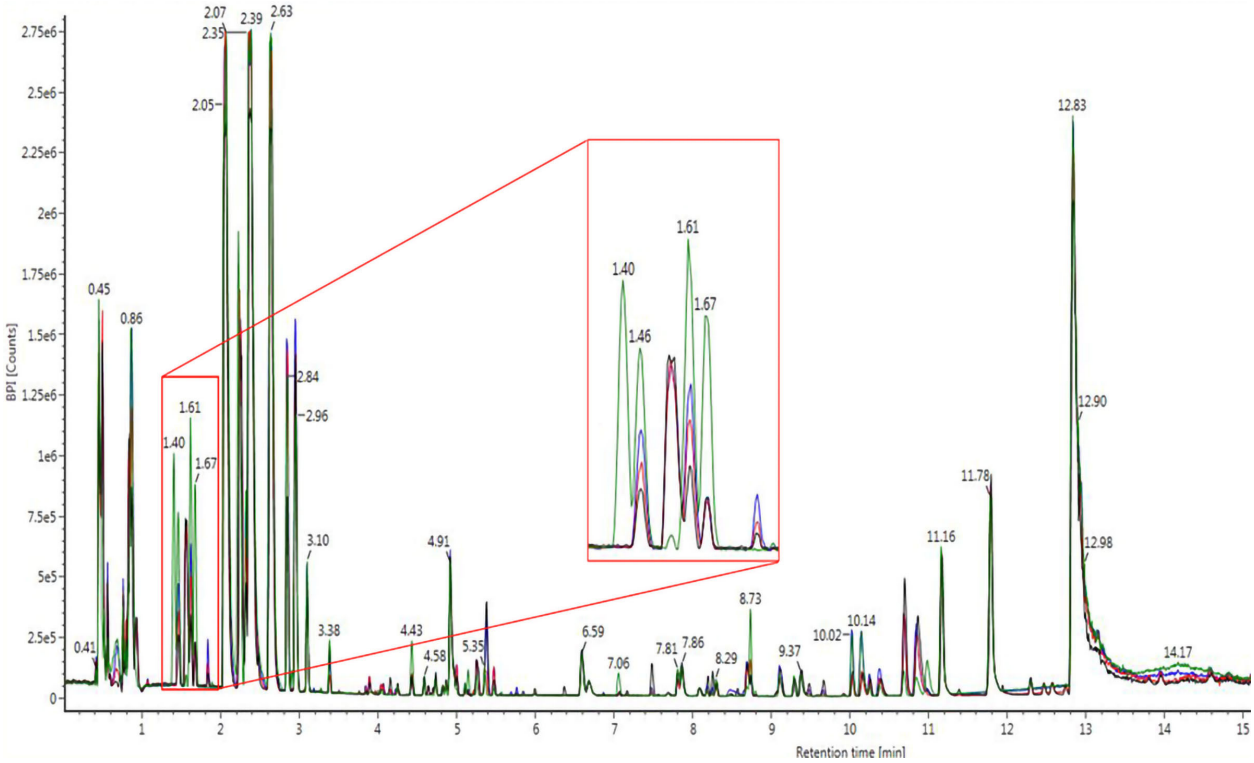

Figure 3. The overlaid LCMS chromatogram of the *Allium sativum* Linn: Ilocos Pink (IPG) groups and the Ilocos Native. Each color corresponds to a specific garlic group: [black] IPG light, [red] IPG moderate, [blue] IPG heavy, and [green] Ilocos Native. The distinct difference between IPG groups and Ilocos Native was found at retention time 1.40 (inset figure: region of distinct retention time between the IPG groups and Ilocos native).

Table 3. Putative compounds found in IPG and *Allium sativum* Linn: Ilocos Native garlic (control).

RT (min)	Putative identification	m/z	Adducts	Library	RT (min)	Putative identification	m/z	Adducts	Library
0.39	Candidate mass 113.9634	113.9634			4.42	Candidate mass 284.0770	284.0770		
0.46	Arginine	175.1189	+H, +Na, +K	TCM	4.56	Candidate mass 988.3616	988.3616		
0.50	Candidate mass 258.1104	258.1104			4.63	Protodiosgenin	595.3842	+H	TCM
0.56	4-O-β-D-Glucopyranosyl fagomine	332.1344	+Na	TCM	4.70	Protodiosgenin	595.3844	+H	TCM
0.66	L-Glutamyl-S-methyl-L-cysteine	265.0855	+H	In-house	4.82	Xanthoxoline	294.0650	+Na	TCM
0.76	4-O-β-D-Glucopyranosyl fagomine	332.1343	+Na	TCM	4.92	Timosaponin G	757.4368	+H, +Na, +K	TCM
0.81	Candidate mass 136.0751	136.0751			4.97	Terrestrosin G	1121.5371	+Na, +H, +K	TCM
0.85	Adenosine	268.1039	+H	In-house	5.25	Tigogenin-3-O-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside	741.4406	+H, +Na	TCM
0.86	Lusianthridin	265.0853	+Na	TCM	5.36	Tigogenin-3-O-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside	741.4410	+H, +K	TCM
0.93	Guanine	152.0563	+H	TCM	5.47	Candidate mass 308.0806	308.0806		

Table 3. Continued . . .

1.45	N-gamma-L-Glutamyl-L-methionine	279.1004	+H, +K	In-house	5.99	Balanophonin	357.1329	+H	TCM
1.55	Candidate Mass 120.0805	120.0805			6.93	2,3-Dimethylphenol	145.0644	+Na	TCM
1.62	gamma-L-Glutamyl-S-(2-carboxy-1-propyl) cysteinylglycine	394.1279	+H, +Na, +K	In-house	7.47	Timosaponin A-1	579.3891	+H, +Na	TCM
1.67	gamma-Glutamyltyrosine	311.1238	+H, +Na	In-house	8.17	Isoeruboside B	1103.5263	+Na, +H, +K	TCM
2.05	L-γ-Glutamyl-S-allyl-L-cysteine	291.1007	+H, +Na, +K	In-house	8.24	Terrestrosin J	1103.5262	+Na, +H, +K	TCM

Table 3. Putative compounds found in IPG and *Allium sativum* Linn: Ilocos Native garlic (control) [continued].

RT (min)	Putative identification	m/z	Adducts	Library	RT (min)	Putative identification	m/z	Adducts	Library
2.23	L-Phenylalanine	188.0702	+Na	In-house	8.46	Candidate mass 230.2472	230.2472		
2.30	Candidate mass 141.9583	141.9583			8.56	Isoeruboside B	1103.5262	+Na, +H, +K	TCM
2.36	Candidate mass 291.2765	291.2765			8.67	Candidate mass 316.2846	316.2846		
2.47	N-gamma-Glutamyl-S-propylcysteine	293.1155	+H, +Na, +K	In-house	8.73	Candidate mass 1145.5376	1145.5376		
2.63	1,7-Bis(4-hydroxyphenyl)-hepta-4E,6E-dien-3-one	295.1288	+H, +Na, +K	TCM	9.47	Protogracillin	1087.5312	+Na, +K	TCM
2.84	Candidate mass 450.1362	450.1362			9.65	Neotigogen-in-β-D-glucopyrano-syl(1→2)-[β-D-xy-lopyranosyl (1→3)]-β-D-glucopy-ranosyl(1→4)-β-D-ga-lactopyranoside	1057.5197	+Na, +K	TCM
2.94	Isatan A	380.0945	+H, +Na, +K	TCM	10.02	Candidate mass 478.2925	478.2925		
3.09	L-gamma-Glutamyl-S-allylthio-L-cysteine	323.0730	+H, +Na	In-house	10.14	1-Linoleoylglycero-phosphocholine	520.3396	+H, +Na, +K	In-house
3.38	5α,11-Dihydroxy-eudesm-3-en-2-one	275.1597	+Na	TCM	10.24	Candidate mass 478.2925	478.2925		
3.38	Nardosinonediol	275.1597	+Na	TCM	10.37	1-Linoleoylglycero-phosphocholine	520.3395	+H, +Na	In-house
4.03	Candidate mass 260.0772	260.0772			10.68	Candidate mass 454.2926	454.2926		
4.15	Oxymaistemonine	454.1857	+Na	TCM	10.84	Candidate mass 496.3398	496.3398		
					10.96	Candidate mass 284.3311	284.3311		

Legend: [TCM] traditional Chinese medicine

DISCUSSION

Allium sativum Linn: Ilocos Pink is a Philippine garlic variety known for its distinct pink color in the bulb skin. Differences in the intensity of this pink color may raise concerns about the authenticity and uniformity of the samples. In this study, we presented a way of categorizing the biochemical and genetic composition of IPG based on this morphological variation.

Variations in the brown pigmentation on the outer skin of IPG were identified using ImageJ analysis. These variations in the outer skin pigmentation are influenced by environmental factors such as temperature, soil composition, and humidity (Hoogerheide *et al.* 2017; Atif *et al.* 2020). ImageJ analysis has been used in plant biology not only in measuring the size and length of a specific plant species but also in assessing its phenotypical characteristics and health status (Polder *et al.* 2012). It is also used in determining the extent of plant tissue damage (Pride *et al.* 2020), calculating the canopy area in plant diversity studies (Concepcion *et al.* 2020), and describing the degree of unique pigmentations in plants (Zhu *et al.* 2021). Indeed, it is well-established that ImageJ is a powerful tool in both plant species characterization and diversity studies.

The SRAP analysis revealed an updated set of marker pairs that can be used to molecularly characterize IPG and its subgroups. In a previous report by Bigtas *et al.* (2020), only ME1-EM3 and ME3-EM3 marker pairs can molecularly describe IPG. However, current findings suggest 20 SRAP marker pairs to molecularly characterize IPG. The addition of new SRAP marker pairs characterizing the IPG and its potential subgroups increases the utility of SRAP in the molecular mapping of IPG, as well as species identification of authentic samples. Likewise, despite the limited literature on its limitations, SRAP cannot yield heterozygosity descriptors and may not be advantageous in examining taxonomic placements affected by selective pressure (Robarts and Wolfe 2014).

Metabolite profiling using liquid chromatography–mass spectrometry (LC-MS) applied the comparison of IPG chromatogram results with reference to the control. Unfortunately, the major peaks in the three groupings of IPG garlic and even in the control group are all similar. However, with the help of PCA and Unifi's Marker Matrix tool, sample groupings can be discriminated and potential markers expressed as EMRT can be generated. The combination of LCMS metabolite profiling and PCA as a technique has been a useful tool in the characterization of a wide variety of samples for various applications such as the authentication of cosmetic products (Cabovska 2015), herbal medicines (Xie *et al.* 2022), and differences among species (Qi *et al.* 2021). In the study of Levandi *et al.* (2014), the method was able to distinguish the changes in oligosaccharide contents as a result of farming methods and seasonal variation, whereas the geographical differences and their effects on metabolic profiling were revealed using the same technique used by Younis and colleagues (2022).

The absence of a distinct IPG metabolite that can be used as a chemical marker for its quality and authenticity assessment is still a major concern. To note, the proposed chemical markers were selected based not on their uniqueness *per se* but rather on their relatively higher abundance than the other groupings. In addition, the identification of metabolites can be hindered by the utilized chemical library and this may affect the further characterization of the analyzed samples. While the multivariate analysis of the LCMS data was able to initially provide a lead that the moderate and heavy groups may be grouped as one, as evidently shown by their clusters, the need for actual markers that discriminate one from the other remains crucial.

It is worth noting that seven out of the 13 proposed markers were saponins – namely, timosaponin G (RT 4.92), terrestrosin G (4.97), timosaponin A-1(RT 7.47), isoeruboside B (RT 8.17), terrestrosin J (RT 8.24), protogracillin (RT 9.47), and neotigogenin-β-D-glucopyranosyl(1→2)-[β-D-xylopyranosyl(1→3)]-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside (RT 9.65). Interestingly, the majority of these putatively identified saponins have not been reported to be present in *Allium sativum* except the eruboside B (Younis *et al.* 2022). Some of these putatively identified saponins have been investigated for their medical applications. Isoeruboside B was found to have potential anticoagulant and pro-fibrinolytic effects *in vitro* (Waller and Yamasaki 2013). Timosaponins and terrestrosins were also known for their cancer cytotoxicity and tumor-suppressing activities, especially the timosaponin A3 (Liao *et al.* 2023; Wei *et al.* 2014), and in ameliorating imiquimod-induced psoriasis-like skin lesions in murine models by terrestrosins D (Guo *et al.* 2022). It is important to note that the majority of the putatively identified saponins from IPG are timosaponins and terrestrosins, and it is recommended to explore these compounds for their anticancer, antifibrinolytic, and anti-inflammatory activities. Aside from this, in terms of its culinary applications, several studies have shown the effects of various food processing conditions on the saponin content of *Allium* species. During the process, saponins are degraded and transformed into other chemical structures as a result of the enzymatic alterations between the sugar chains and aglycones and hydrolysis (Kregiel *et al.* 2017). Interestingly, saponins from *Allium sativum* have more stable chemical properties, which are more suitable for cooking and storage than their organosulfurs (Luo *et al.* 2011). Likewise, this is an opportunity to explore the stability of saponins derived from the IPG and its antioxidant properties following the food process.

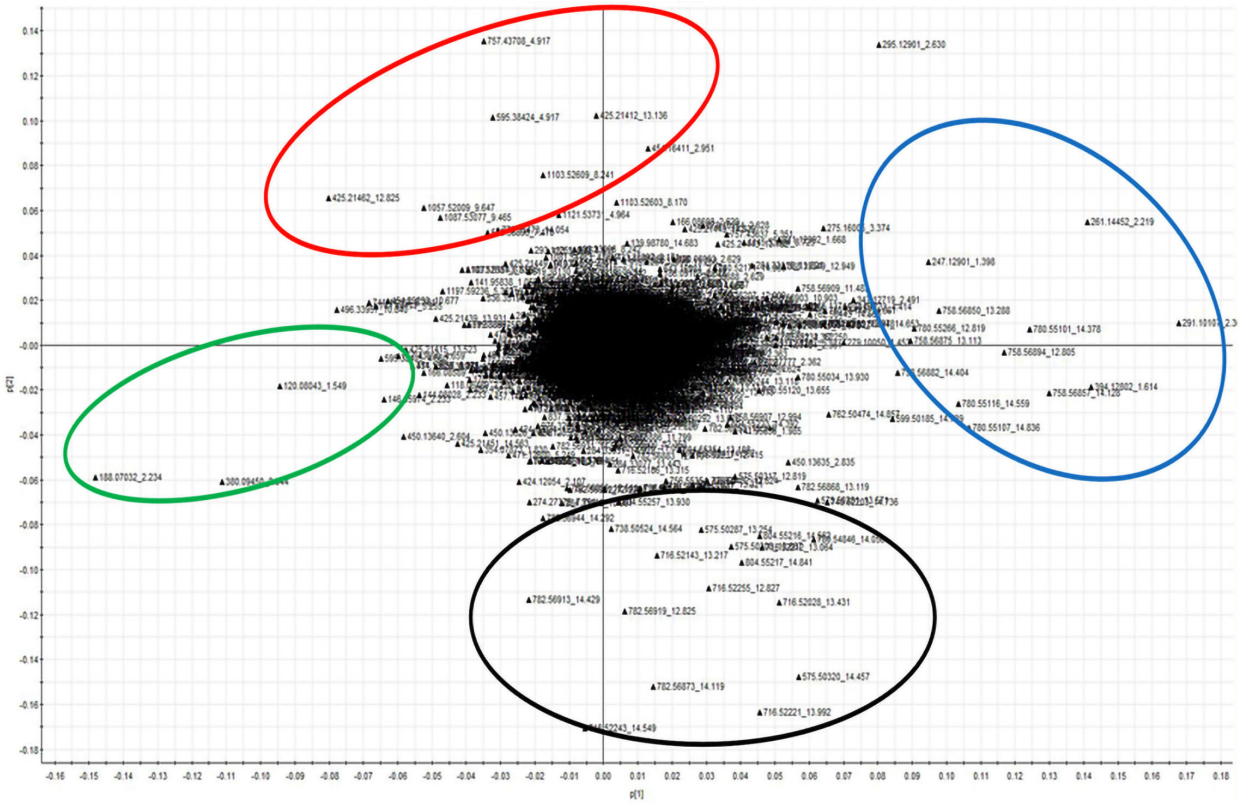

Figure 4. Loading plot correlated from principal component analysis (PCA) of the *Allium sativum* Linn: Ilocos Pink (IPG) with the encircled potential markers. Each color corresponds to a specific garlic group: [red] IPG light, [green] IPG moderate, [black] IPG heavy, and [blue] Ilocos Native.

Table 4. Proposed chemical markers of the *Allium sativum* Linn: Ilocos Pink (IPG) groups based on principal component analysis (PCA) and Unifi's "marker matrix" tool.

Group	m/z	RT	Concentration		
			Light	Moderate	Heavy
IPG light	757.4371	4.92	High	Low	Low
	595.3842	4.92	High	Low	Low
	1103.526	8.24	High	Low	Low
	1057.52	9.65	High	Low	Low
	1087.531	9.47	High	Low	Low
	1103.526	8.17	High	Low	Low
	1121.537	4.96	High	Low	Low
	779.5147	14.05	High	Low	Low
	579.789	7.48	High	Low	Low
IPG moderate/ IPG heavy	716.5203	13.43	Low	Mid	High
	716.5214	13.22	Low	High	High
	780.5485	14.06	None	Low	High
	575.5029	13.25	None	Low	High

The levels of saponin in garlic are affected by several environmental factors, agronomic conditions, and post-harvest treatments. In a study by Abdelrahman *et al.* (2021) quantitative levels of saponins in garlic are affected by agroclimatic changes. However, in conditions where garlic samples are grown in the same geographic location and seasonal pattern, other factors affecting the levels of saponin content must be considered. One factor to consider is the degree of the maturation of the plant, as reported in the study by Ferreira *et al.* (2011), which showed that young leaves of *Brachiaria brizantha* had higher saponin concentration than the matured ones. As this study revealed the IPG light group to have the highest saponin content, the evidence as to the inverse correlation between saponin content and bulb skin pigmentation intensity is yet to be established. As pointed out by Volk and Stern (2009) in their garlic phenotypic study, varieties grown in diverse conditions have "highly elastic soil nutrient responses, particularly relating to skin color and yield."

With all these somewhat contradicting results on the molecular characterization, it should be noted that the chemical profiling done was limited to methanolic extracts only. With this, it is suggested that other solvents of different polarities may be used in the discovery of chemical markers as they may not have been captured in the current study.

CONCLUSION

SRAP analysis provided a robust system for discriminating IPG and its subgroups in terms of distinct primer pairs. Metabolite profiling using LC-MS revealed 13 proposed metabolite markers to discriminate IPG and its subgroups to Ilocos Native, but this characterization is based on the relative abundance. The majority of the observed metabolite markers were saponins but it is still unknown whether it affects the degree of brown pigmentation on the *Allium sativum* skin. Furthermore, evaluation of the putatively identified saponins and their biochemical interactions is recommended in order to understand their mechanisms *in vitro*. The use of other solvents of different polarities is also recommended to assess the metabolite profile of IPG and its subgroups.

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STATEMENT ON CONFLICT OF INTEREST

The authors declare no conflict of interest.

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