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Article

# Metabolomic Changes in Rice (*Oryza sativa* L.) Subjected to Herbicide Application through HPLC-HRMS and Chemometrics Approaches

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**ABSTRACT:** This study evaluated rice samples (*Oryza sativa* L.)—rice husk, husk and grain, polished grain, and unpolished grain—exposed to imazapyr, imazapic, and clomazone using high-performance liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS) and chemometric analysis. Partial least squares discriminant analysis (PLS-DA) was applied to HPLC-HRMS data, successfully distinguishing between herbicide-treated and control samples. Additionally, variable importance in projection (VIP) scores were then computed to identify key metabolites contributing to class differentiation, with higher scores indicating the most influential *m/z* values. These findings revealed metabolites affected by herbicide exposure and variations in the rice matrix. Furthermore, the most relevant *m/z* values were putatively annotated using spectral libraries, enabling the assessment of herbicide-induced metabolomic changes in rice. Herbicide treatment resulted in reduced free sugar levels across all rice matrices and led to a decrease in flavonoid content in the husk, indicating a potential suppressive effect on flavonoid accumulation. In addition, the herbicide treatment markedly disrupted the phenylpropanoid biosynthesis pathway. Overall, the combination of HPLC-HRMS analysis with multivariate approaches proved effective in detecting significant variations in the rice metabolome cultivated under herbicide application, paving the way for understanding the effects of herbicides in crop cultivation.

**KEYWORDS:** *imazapyr, imazapic, clomazone, herbicides, rice metabolome, multivariate analysis*



## 1. INTRODUCTION

Food security is currently a pressing issue in light of an unprecedentedly growing global population.[1,2] In this context, rice (*Oryza sativa* L.) stands out as an important and nutritious food for at least half of the world's population. Moreover, rice production plays an important role in the economies of several countries. Due to the demand for the production of this grain, strategies have been implemented to enhance productivity and ensure high yields, which includes the use of chemical agents to control pests and weeds.[1−3]

The presence of weeds in rice cultivation reduces rice yield and negatively impacts the grains' quality. Weeds may compete with rice for nutrients, water, and space, compromising the rice cultivation.[4−6] Consequently, the use of chemical agents such as herbicides is vital to protect cultivation. The mixture of herbicides or their sequential applications is a normal rice producer practice, aiming for effective and comprehensive control of weeds in rice crops.[7] In this scenario, herbicidal agents such as imazapic, imazapyr and clomazone are the most widely used combination in rice cultivation in Brazil.[8,9] Imazapic and imazapyr are herbicides belonging to the imidazolinone chemical class, which acts via inhibition of the acetolactate synthase enzyme that disrupts the biosynthesis of branched-chain amino acids essential for weed growth. The combination of imazapic and imazapyr enhances the herbicidal spectrum, once imazapyr inhibits perennial grasses and sedges growth and imazapic controls the incidence of broadleaf weeds.[10−12] Additionally, clomazone is an isoxazolinonase herbicide that controls the occurrence of annual grass and broadleaf weeds.[13,14] Overall, Imazapyr and imazapic are selective herbicides effective against a broad spectrum of terrestrial and aquatic weeds, while clomazone is widely used for the control of terrestrial weeds.[15,16]

Despite offering many advantages in increasing grain productivity and quality, these herbicides may pose risks regarding human toxicity if not handled properly. Moreover, herbicides can influence plant metabolism, which could result in different secondary metabolites.[5,7] For instance, the literature indicates that clomazone can disrupt the synthesis of carotenoids, an essential compound class, intrinsically related to protecting chlorophyll from photooxidation, which absence could result in leaf bleaching and plant death.[4,14]

Hence, it is essential to develop studies on the metabolomic profile of plants exposed to chemical agents. In this regard, untargeted metabolomic assays are an effective tool for

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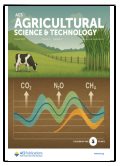





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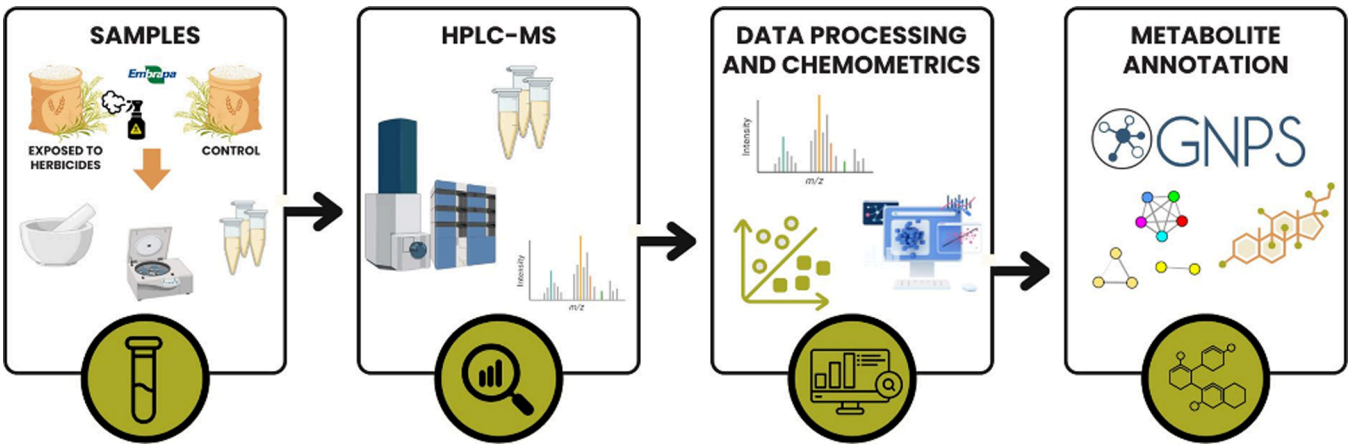


**Figure 1.** Analytical protocol of the metabolomic study of rice samples.

deciphering variations arising from specific conditions, such as the exposure of plants to herbicides.[17−19] High-resolution mass spectrometry (HRMS) stands out as an analytical technique widely applied to this aim due to the ability to deliver high information content and accuracy on metabolite identification.[20−22] The chromatographic systems have been integrated into metabolomics methods to address matrix complexity and prevent ion suppression effects in HRMS systems. These strategies are well established in the literature and consistently contribute to the enhancement of reproducibility, sensitivity, specificity and MS resolving power.[20,23]

Among the challenges of metabolomics through HRMS, data processing is impractical without the aid of advanced tools due to the sheer volume of information. Thus, chemometric approaches are invaluable in metabolomics studies, as they provide statistical and machine-learning strategies capable of elucidating variations in the metabolome of the organism under study.[24,25] Therefore, this study aims to evaluate the effects of using imazapyr, imazapic, and clomazone in rice cultivation through high-performance liquid chromatography coupled with HRMS (HPLC-HRMS) and chemometric approaches (Figure 1). Combined, these approaches contribute to revealing the molecular-level impacts of these chemical agents, which are essential in rice cultivation but may pose risks to consumers and alter the nutritional value of globally consumed foods.

## 2. MATERIALS AND METHODS

**2.1. Chemicals and Reagents.** Methanol HPLC-grade (>99.9%) was purchased from Tedia Company (Fairfield, USA) and formic acid (≥98.0%), was purchased from Sigma-Aldrich (St. Louis, USA). Ultrapurified water was obtained from MS2000 WFI equipment (Gehaka, São Paulo, SP, Brazil). Clomazone (≥98%), imazapic (≥98%), and imazapyr (≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**2.2. Samples.** The plants were grown at Embrapa Arroz e Feijão (Santo Antônio de Goiás/GO) in 10-L pots, cultivating the reference samples (control - C) and the samples cultivated with herbicides (CH). The herbicides were diluted in water - clomazone at 96 g $L^{-1}$, imazapyr and imazapic at 6.3 g $L^{-1}$ - were applied to rice seeds via seed treatment prior to sowing. The solution was uniformly sprayed onto the seeds using a hand sprayer while continuously mixing to ensure total coverage. The treated seeds were air-dried at room temperature for 1 h before planting.

Grain samples were collected approximately 120 days after seedling emergence. Three plants were sampled three times to their biological replicates, generating nine samples overall. The samples were cultivated under the same conditions, including soil composition, humidity, solar light exposure, and temperature. The collected samples were divided into four distinct matrices: rice husk (H), husk and grain (HG), polished grain (PG), and unpolished grain (UG). A grain huller was used for the husk and the grain separation. The polishing of the grains was performed using the same equipment. All these matrices were ground into a fine powder using an analytical mill (IKA, A11) and stored in glass containers with lids under ambient light, temperature, and humidity conditions.

The extraction of rice metabolites was adapted from the protocol described by Zhang et al.[26] which employes cold solvent for extraction. This strategy may improve extraction efficiency by preserving the thermally sensitive nature of various metabolites.[27] A solution of 1 mg.$mL^{-1}$ of these powders was prepared, in methanol. The mixture was vortexed for 1 min and stored at 4 °C for 12 h. After this period, the samples were centrifuged at 15000 rpm and 4 °C in a centrifuge Mikro 200-Hettich (Tuttlingen - Germany) for 15 min and the supernatant was collected as filtered (PTFE 0.22 $\mu$m, Whatman) for HPLC-HRMS analysis.

**2.3. HPLC-HRMS Analysis.** HPLC-HRMS analyses were performed on an LC-20AD system (Shimadzu, Kyoto, Japan) coupled with a microTOF-Q III mass spectrometer (Bruker, Bremen, Germany). Chromatographic separation was achieved using a C18 column (2.1 × 100 mm, 1.8 $\mu$m) with a column temperature of 40 °C, a sample injection volume of 3 $\mu$L, and a mobile phase flow rate of 0.250 mL $min^{-1}$ in isocratic elution mode. The mobile phase consisted of methanol and 0.10% (v/v) formic acid in water (80:20). The total chromatography run took 45 min. HRMS analysis was conducted in positive ionization mode using an electrospray ion source, with a capillary voltage of 4 kV, a drying gas flow rate of 8 L.$min^{-1}$, a nebulizer pressure of 4 bar, and a capillary temperature of 180 °C. Mass spectra were archived ranging from 100 to 1200 $m/z$ and processed using the Data Analysis software version 5.0 (Bruker Daltoniks, Bremen, Germany).

**2.4. Data Processing.** The HPLC-HRMS files were exported in *.mzXML* format from Data Analysis software and imported into MATLAB 2024a (MathWorks, Natick, MA, USA) for chemometric analyses. The data were initially aligned concerning retention time and $m/z$ to ensure consistency and accuracy across the data set. This alignment process is crucial for correcting any variations in retention time and $m/z$ that may arise during the analytical run, which could otherwise obscure true biological differences. The aligned data were then subjected to further preprocessing steps, including normalization and peak detection, to prepare for comprehensive metabolomic analysis.

In the data processing workflow, peak heights were utilized to construct the spectral matrix for subsequent chemometric analysis. After aligning the HPLC-HRMS data concerning retention time and $m/z$, peak detection was performed across the 72 samples. For each $m/z$ value, the maximum intensity observed within the aligned


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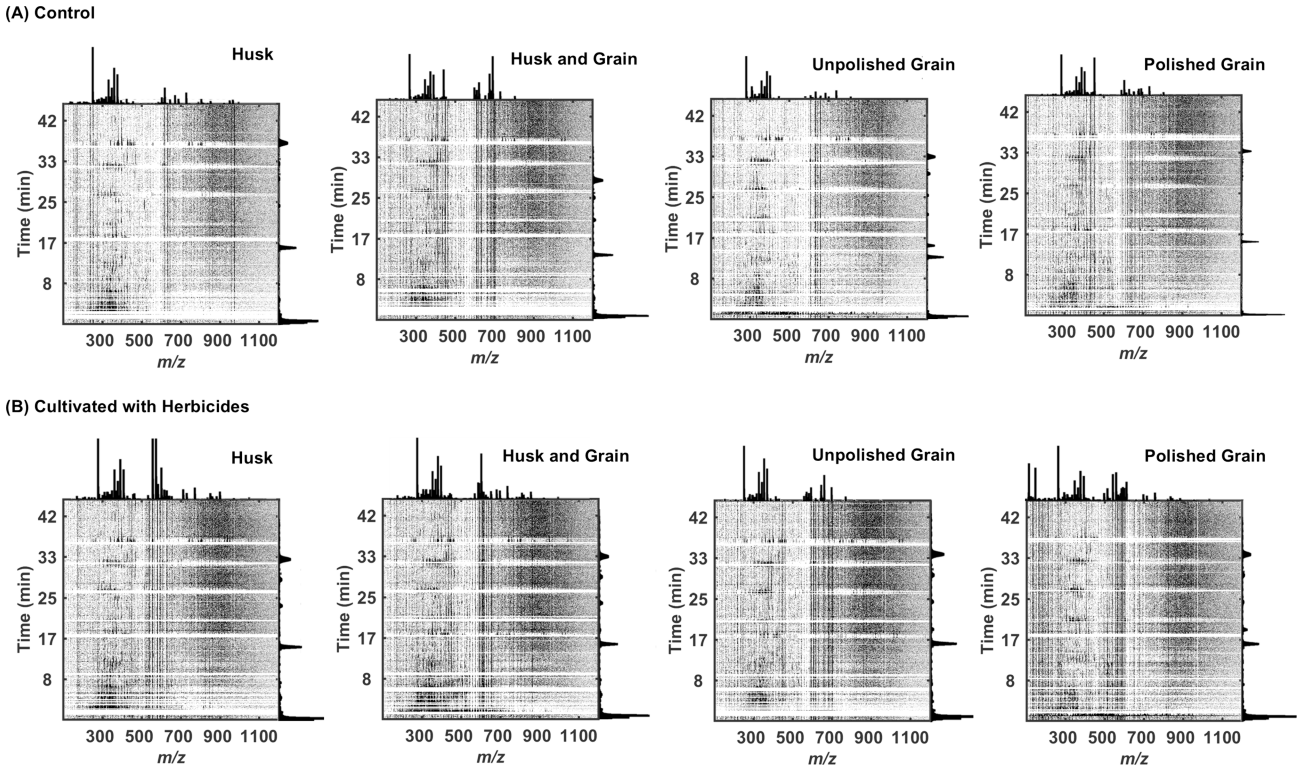


**Figure 2.** Grayscale dot plot representing HPLC-HRMS data for control samples (A) and herbicide-treated samples (B).

retention time window was recorded as the peak height. When a specific $m/z$ value had no detected intensity in each sample, a small value ($10^{-16}$) was imputed to avoid gaps in the matrix without introducing excessive zeros. This approach captures the highest signal corresponding to a metabolite, providing a consistent measure across samples. The resulting data matrix, composed of peak heights for each $m/z$ across all samples, served as the foundation for subsequent statistical and machine-learning analyses, enabling the investigation of metabolomic variations associated with herbicide exposure. Autoscaling was applied to all data before performing chemometric analysis.

**2.5. Chemometric Approaches.** Chemometric approaches were performed using MATLAB 2024a (MathWorks, Natick, MA, USA). Unsupervised and supervised chemometric analyses were applied to investigate the metabolomic differences in rice samples subjected to herbicide application. Before chemometric analyses, the data set was normalized by sum and autoscaled using mean-centering and unit variance scaling. Initially, Principal Component Analysis (PCA)[28] was performed as an exploratory tool to evaluate the natural variability and clustering in the data set. This unsupervised analysis provided a preliminary visualization of the data structure, revealing patterns and cluster trends among samples (control vs herbicide) and rice matrix kind (husk, polished grain, unpolished grain, husk-and-grain).

Following the exploratory PCA, Partial Least Squares Discriminant Analysis (PLS-DA)[29] was employed as a supervised technique to enhance the classification of predefined groups and to identify the variables that contribute most significantly to the discrimination between classes. The PLS-DA analysis was conducted in three stages. First, a model was built to contrast all herbicide-treated samples against the control samples, aiming to identify broad metabolomic differences induced by herbicide exposure (2 classes). In the second stage, PLS-DA was applied to distinguish between the four rice matrices (husk, polished grain, unpolished grain, and husk-and-grain), independently of treatment, providing insights into metabolomic variations related to the structural and compositional differences of each rice part (4 classes). Lastly, PLS-DA was performed on the full data set, considering both treatment and rice part simultaneously, resulting in eight classes (control and herbicide-treated for each of the four rice matrices). This comprehensive model allowed for a detailed evaluation of the interaction between herbicide treatment and rice matrix on the metabolomic profile.

This stepwise modeling approach was chosen to capture not only isolated effects of herbicide treatment or rice matrix but also their combined impact, enabling the detection of potential interactions and matrix-specific herbicide responses within a unified framework.

Additionally, the Variable Importance in Projection (VIP)[30] scores were utilized to rank the metabolites based on their contribution to the model's discriminatory power. These scores are crucial for identifying key metabolites facilitating a deeper understanding of the biochemical pathways impacted. Together, these analyses allowed for the validation of the robustness and discriminatory power of the PLS-DA models, ensuring reliable classification and aiding in the selection of significant variables that could contribute to understanding the metabolomic effects of herbicide exposure in rice samples.

Model performance was assessed using key classification metrics, including sensitivity, specificity, accuracy, and precision. Sensitivity measured the model's ability to correctly identify samples within each class (true positive rate), while specificity assessed the model's ability to exclude samples from other classes (true negative rate). Accuracy provided an overall measure of correctly classified samples across all classes, and precision evaluated the reliability of the model in identifying each class without false positives. Cross-validation was performed to assess model reliability and prevent overfitting, using a stratified 10-fold approach to ensure balanced class representation across folds.

**2.6. Putative Metabolite Annotation.** The data acquired from HPLC-HRMS/MS analyses were converted to.*mzML* format using MSConvert software (ProteoWizard, Palo Alto, CA, US). This study leveraged metadata to organize compound information according to the Global Natural Products Social Networking (GNPS) online workflow. Metabolite annotations were achieved by searching the experimental mass spectra (MS/MS) against the GNPS spectral library, utilizing tools such as classic Molecular Networking (MN),[31] Feature-Based Molecular Networking (FBMN),[32] Dereplicator+,[33] Network Annotation Propagation (NAP),[34] MolDiscovery,[35] MS2LDA,[36] and MolNetEnhancer,[37] along with chromatographic data analysis, including retention time.



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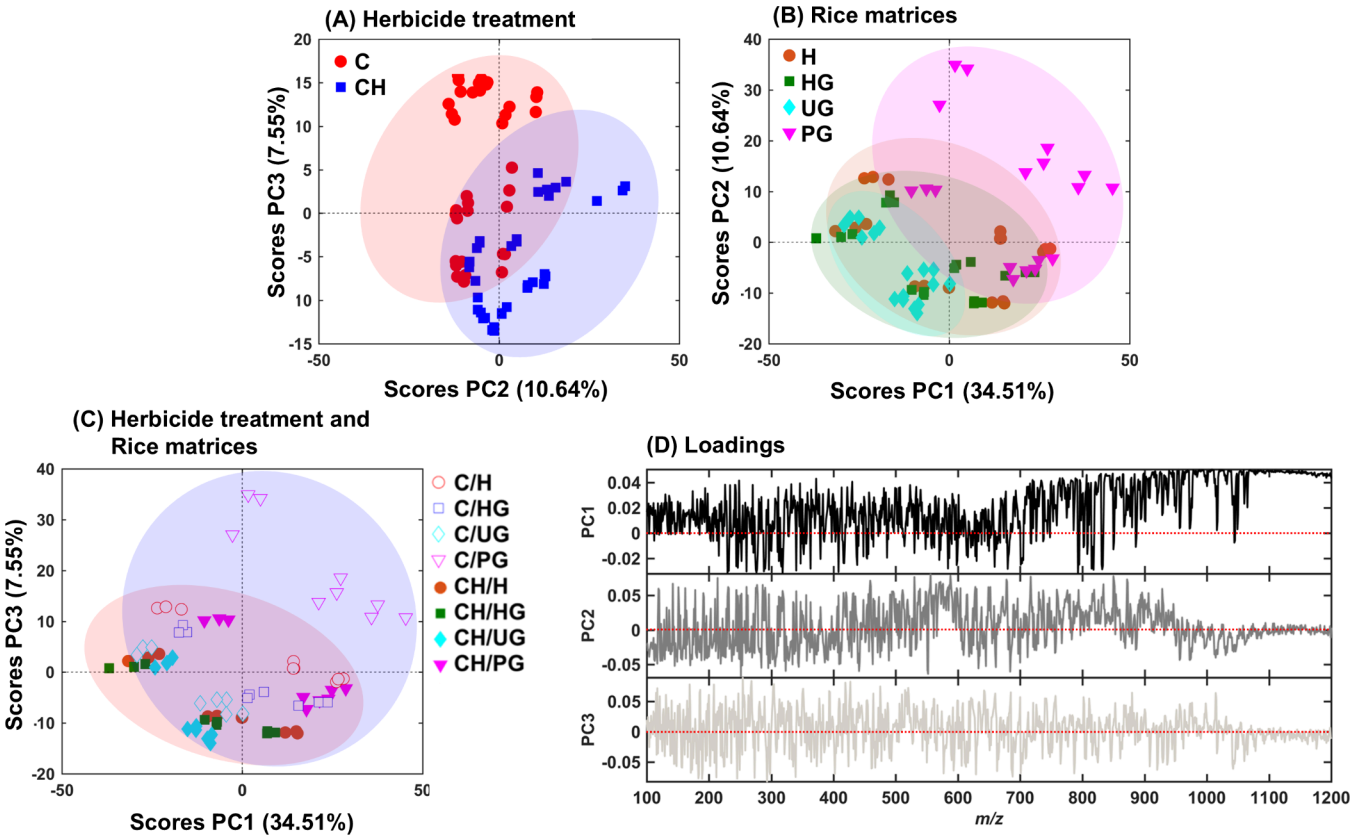


**Figure 3.** PCA scores for herbicide-treated and control rice samples, separated by (A) treatment group (C: Control, CH: Cultivated with Herbicide), (B) rice matrix (H: Husk, HG: Husk and Grain, UG: Unpolished Grain, PG: Polished Grain), and (C) combined treatment and rice matrix groups. Panel (D) shows the loadings plot for PC1, PC2, and PC3, indicating the contribution of each $m/z$ variable to the principal components.

## 3. RESULTS AND DISCUSSION

**3.1. General Overview of HPLC-HRMS Data.** Rice is one of the most widely consumed grains globally, valued for its nutritional content, including essential amino acids (cysteine, tryptophan, histidine, and arginine), carbohydrates, flavonoids and vitamins, as well as therapeutic compounds such as vitamin E, ferulic acid, gamma oryzanol, and salicylic acid.[2,38] Flavonoid compounds found in rice exhibit antioxidant properties and therapeutic effects, including the reduction of blood glucose levels and cholesterol absorption, and may be associated with a decreased risk of cardiovascular diseases.[39] The nutritional diversity of rice underscores the need to evaluate how cultivation-related factors may impact its nutritional value, such as the use of herbicidal agents during its cultivation. With the aim of increasing grain production, the use of herbicides in cultivation has become essential, and herbicides such as glyphosate, propanil, 2,4-D, clomazone, imazapyr, and imazapic are commonly employed. These chemical agents can contribute both to the enhancement of crop yield and to the rice metabolome, which may affect its nutritional quality.[6,8,9,40] When compared to glyphosate, the use of herbicides such as imazapyr, imazapic, and clomazone is preferable due to their selectivity, which allows effective weed control without harming the crop.[40,41]

Therefore, this study aimed to assess the potential impact of clomazone, imazapyr, and imazapic on the rice metabolome, examining both grains (polished and unpolished) and husks to ensure a comprehensive analysis. By comparing control samples (C) with those treated with herbicides (CH), it was possible to observe variations in the metabolite profiles indicative of the molecular-level effects of herbicide application on rice. Aiming for a comprehensive assessment of the adverse effects of these herbicides on rice cultivation, different matrices such as the grain and husk were analyzed. These matrices exhibit distinct metabolic profiles and can thus provide complementary information regarding the changes induced by external conditions.

Figure 2 presents a two-dimensional dot plot of HPLC-HRMS data for each sample type, illustrating the distribution of detected signals across retention times and $m/z$ values for control and herbicide-treated samples. The grayscale scale reflects the presence or absence of signals, highlighting differences in feature detection patterns between the two groups rather than relative intensity. Additionally, the figure includes overlays of the mass spectrum (top) and chromatogram (right), allowing for an integrated view of the spectral and chromatographic profiles. These visualizations reveal distinct intensity patterns across different rice matrices (husk, husk and grain, unpolished grain, and polished grain), highlighting how herbicide treatment influences metabolite presence and distribution across the rice components.

In the HPLC-HRMS plots presented in Figure 2, visual differences can be observed in the detected signals of control samples and herbicide-treated samples, with noticeable variations around $m/z$ 300, 500, and 700 in both husk and grain samples. These areas suggest an increased abundance of certain metabolites, probably as a response to herbicide exposure. For instance, regions with increased intensities in


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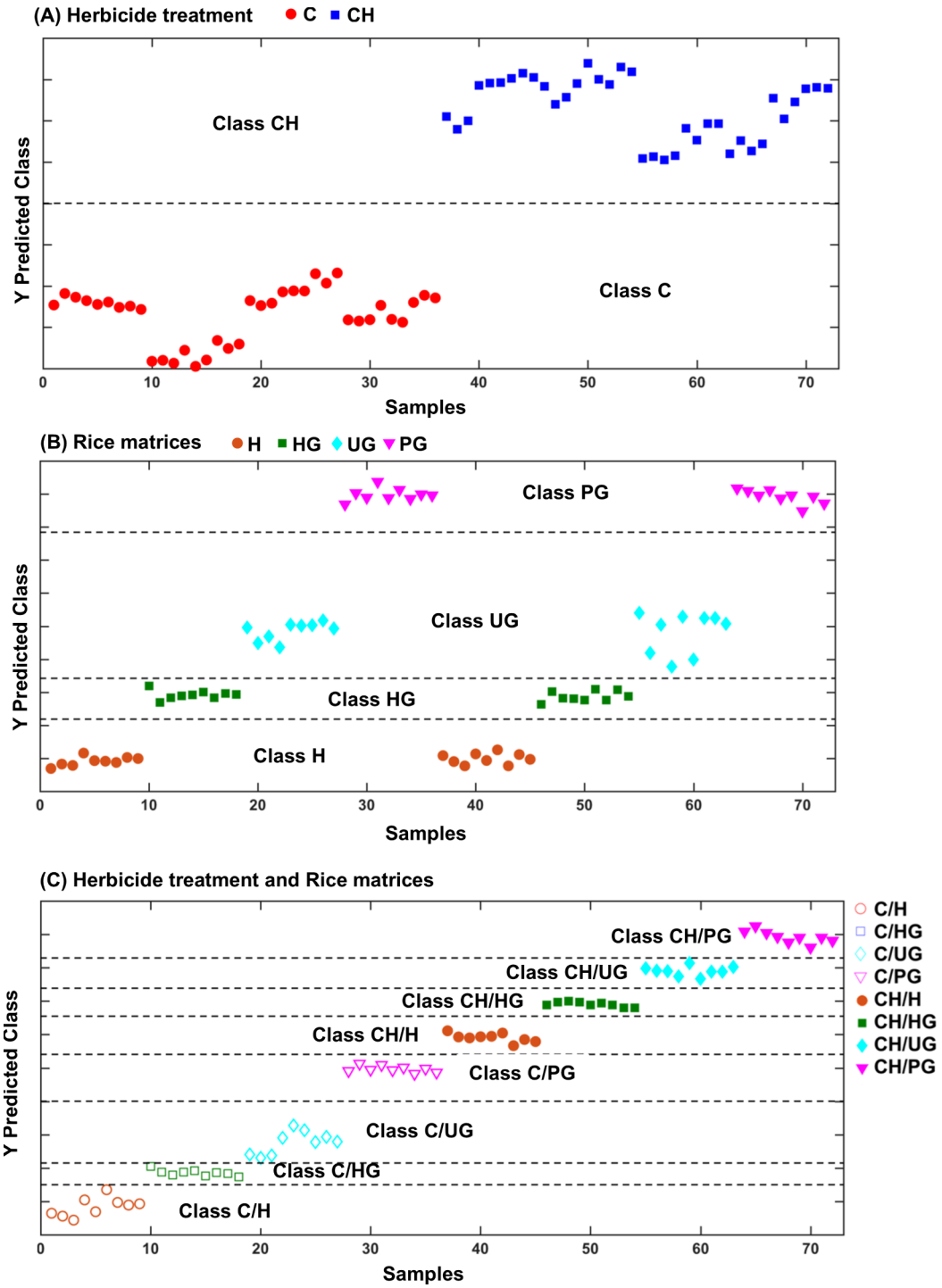


**Figure 4.** Predicted classification plots from PLS-DA models for (A) herbicide treatment (C: Control, CH: Cultivated with Herbicide), (B) rice matrices (H: Husk, HG: Husk and Grain, UG: Unpolished Grain, PG: Polished Grain), and (C) combined treatment and rice matrices (C/H, C/HG, C/UG, C/PG, CH/H, CH/HG, CH/UG, and CH/PG). The *Y*-axis represents the predicted class for each sample, illustrating the effectiveness of PLS-DA in separating the different classes with the dashed line representing the threshold.

the treated samples may correspond to metabolic byproducts or stress-related compounds induced by herbicide application, reflecting changes in the metabolic pathways of rice. It is important to highlight that all analyzed samples were cultivated in the same conditions: soil composition, humidity, solar light, and temperature.

The elevated signal intensity in herbicide-treated samples could indicate an upregulation of specific metabolites related to stress responses. Herbicides are known to interfere with essential biosynthetic pathways, potentially leading to the accumulation of intermediate or secondary metabolites as the plant attempts to mitigate the effects of these chemicals. In this context, higher intensities in the treated groups could be linked to compounds involved in detoxification or defense mechanisms, such as antioxidants or phenolic compounds, which the plant may produce in response to oxidative stress induced by


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herbicide exposure.[42,43] Furthermore, the distinct intensity patterns observed in different rice matrices (husk, unpolished grain, and polished grain) suggest that the effect of herbicides varies across rice components. The husk, for instance, might exhibit higher intensities for metabolites related to protective functions, as it is the outermost layer and may play a role in shielding the grain from external stressors. In contrast, variations within the unpolished and polished grains could be more reflective of changes in primary metabolism, which could impact the nutritional profile of the rice.

These findings underscore the influence of herbicide treatment on the rice metabolome, revealing specific areas of metabolic alteration and suggesting potential biomarkers associated with herbicide-induced stress responses. However, given the large number of variables in the data set, statements regarding variations in these regions must be supported by statistical analyses, in order to determine whether such variations are indeed relevant for distinguishing between control and herbicide-treated samples. Nevertheless, this initial observation warrants further analysis to identify and characterize the specific metabolites contributing to these intensity differences.

**3.2. Chemometric Analysis of Metabolomic Data.** A data matrix used for the analysis consisted of 72 samples and 108,725 $m/z$ features. PCA was performed to explore the overall variance in the data set and assess potential clustering patterns between herbicide-treated and control samples, as well as among the different rice matrices. The first three principal components (PC1, PC2, and PC3) explained approximately 53% of the total variance, indicating a moderate capture of data variability. Figure 3 displays the PCA score plots generated from these components, along with the respective loadings plots.

In Figure 3A, samples are grouped based on herbicide treatment (C and CH). A slight separation is observed between the C and CH samples along the combination of PC2 and PC3, suggesting some differentiation in metabolomic profiles between control and treated samples, though the separation is not definitive. Figure 3B shows the grouping by rice matrix (H, HG, UG and PG). In this case, the groups H, HG, and UG are largely overlapping, with no clear separation, while PG (polished grain) shows a slight distinction with positive scores along both PC1 and PC2, indicating a possible loss of nutrients associated with polishing.

In Figure 3C, where samples are grouped by both herbicide treatment and rice matrix (resulting in eight groups), no clear separation is observed among the combined groups. However, there is a trend indicating a slight separation between control and herbicide-treated samples, as observed in Figure 3A, with filled and empty symbols representing treated and control samples, respectively. This tendency suggests that while the PCA captures some degree of variance associated with herbicide treatment, it is not sufficient for complete discrimination among all groups.

The loadings plot (Figure 3D) presents the contributions of individual $m/z$ variables to the first three principal components. Each line corresponds to the loading weights for PC1, PC2, and PC3, respectively, across the $m/z$ range. Although no specific $m/z$ values showed sharp or isolated contributions, the loading profiles illustrate the complex and distributed nature of the variance across features. Overall, the PCA results suggest that the metabolomic differences induced by herbicide treatment or related to rice matrix are subtle and multifactorial, reinforcing the need for a supervised approach such as PLS-DA to improve class separation and interpretation.

PLS-DA is a supervised chemometric technique employed to assign samples to predefined groups and to predict the class membership of unknown specimens. This method is particularly well suited for the classification and characterization of high-dimensional data sets, such as those provided by HPLC-HRMS.[44] Therefore, PLS-DA was applied to achieve a more effective separation between herbicide-treated and control samples, among different rice matrices. This supervised approach aimed to enhance the discrimination of predefined classes by focusing on variations specifically relevant to each group. The PLS-DA models were developed in three configurations: (A) contrasting herbicide treatment (control, and cultivated with herbicide), (B) analyzing rice matrices individually (husk, husk and grain, unpolished grain, and polished grain), and (C) combining treatment and rice matrices, resulting in eight distinct classes.

Figure 4 shows the predicted classification plots for each configuration, where samples are mapped to their respective predicted classes. In the PLS-DA classification plots, the $y$-axis represents the predicted class values derived from the PLS regression model, where each class is assigned a distinct numerical code. These values indicate the predicted group membership for each sample, allowing direct visualization of the classification performance. Dashed lines separate the class boundaries, illustrating how well the model assigns samples to their correct classes. In Figure 4A, the PLS-DA model effectively distinguishes between control and herbicide-treated (C and CH) samples, with clear separation along the Y-predicted class axis. Figure 4B displays the classification by rice matrices, with each group (H, HG, UG, and PG) showing consistent predictions, though some proximity is observed, particularly for the HG and UG classes. Finally, Figure 4C combines both herbicide treatment and rice matrices, classifying the samples into eight classes (C/H, C/HG, C/UG, C/PG, CH/H, CH/HG, CH/UG, and CH/PG). The dashed black line represents the threshold determined by using a normal probability density function and Bayes decision.[45]

Despite the complexity of this classification, the model shows a good level of separation across the combined groups, reinforcing the ability of PLS-DA to capture specific metabolomic differences associated with both herbicide exposure and rice components. The PLS-DA models achieved perfect classification performance across all three configurations, with accuracy, sensitivity, specificity, and precision values of 1 (or 100%) in each case. This demonstrates the effectiveness of the supervised approach in fully separating all classes. The models were constructed with 3, 13, and 14 latent variables, respectively, for the configurations contrasting herbicide treatment (control vs treated), rice matrices (H, HG, UG, PG), and the combined treatment and matrix groups (eight classes). This progressive increase in the number of latent variables highlights the necessity for a more complex model as the number of classes and classification complexity increases. The results align with the expectation that, as the number of classes grows, a higher number of latent variables is required to capture the additional variability and effectively distinguish between groups. To ensure model reliability and avoid overfitting, all PLS-DA models were internally validated using cross-validation. The consistent perfect classification performance across folds, together with the absence of


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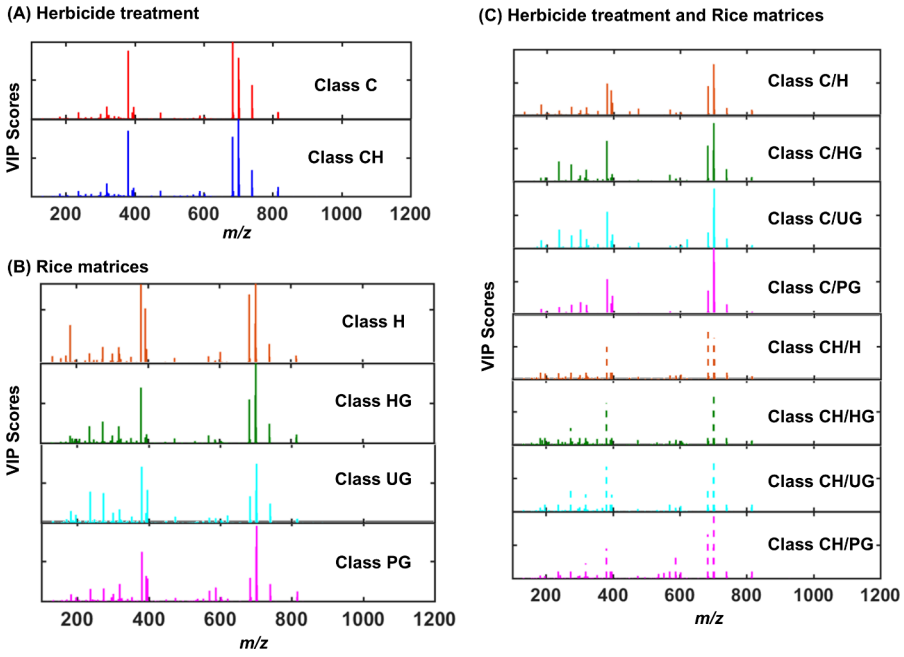


**Figure 5.** VIP score plots from the PLS-DA models, showing the most influential $m/z$ variables for class separation across different configurations: (A) herbicide treatment (C: Control, CH: Cultivated with Herbicide), (B) rice matrices (H: Husk, HG: Husk and Grain, UG: Unpolished Grain, PG: Polished Grain), and (C) combined treatment and rice matrices (C/H, C/HG, C/UG, C/PG, CH/H, CH/HG, CH/UG, CH/PG). The VIP score axes are normalized from 0 to 1 within each model.

misclassifications in the confusion matrices, supports the robustness of the models.

VIP scores were calculated to identify the key metabolites contributing to the separation between classes in each PLS-DA model. At this test, VIP scores rank the $m/z$ variables based on their contribution strength to the model's discrimination, highlighting the most influential features for class distinction. Each VIP score axis is normalized from 0 to 1, allowing the comparison of variable importance across different classes. Figure 5 displays the VIP scores for each model configuration: (A) herbicide treatment (control vs cultivated with herbicide), (B) rice matrices (husk, husk and grain, unpolished grain, polished grain), and (C) the combined model of herbicide treatment and rice matrices (eight classes). Higher VIP scores indicate $m/z$ values that play a more significant role in differentiating the classes within each model, thereby providing insights into the metabolites most affected by herbicide exposure and differences among rice matrices.

In Figure 5A, the PLS-DA model contrasting herbicide-treatment, the $m/z$ values 381, 683, 701, and 739 stand out with the highest VIP scores. These $m/z$ values denote high importance across both classes, being a key contributor to the separation between herbicide-treated and control groups. The fact that these metabolites are prominent in both classes suggests that they may play significant roles in the baseline metabolic composition of rice.

In Figure 5B, which represents the PLS-DA model based on the different rice matrices (H: Husk, HG: Husk and Grain, UG: Unpolished Grain, PG: Polished Grain), the same $m/z$ values observed in the previous model—381, 683, 701 and 739—continue to show high VIP scores, indicating their consistent importance across rice matrices. This reinforces their role as influential metabolites within the rice metabolome, potentially involved in fundamental metabolic pathways that vary with rice component or structural composition.

In addition to these, $m/z$ 393 stands out with a particularly high VIP score in the husk (H) matrix, suggesting its unique significance in this part of the rice grain. The prominence of $m/z$ 393 specifically in the husk may indicate a metabolite that plays a protective or structural role in the outer layer of the rice, potentially involved in defense mechanisms against environmental stressors, such as herbicides. This finding highlights the differentiated metabolic profile of the husk compared to other matrices, suggesting that certain metabolites are more concentrated or have heightened importance in this protective layer.

In Figure 5C, which combines herbicide treatment and rice matrices (resulting in eight classes), the $m/z$ values 381, 683, 701, and 739 still exhibit high VIP scores, underscoring their consistent role in differentiating groups across all configurations. This consistency suggests that these metabolites are central to the rice metabolome, potentially associated with fundamental metabolic pathways affected by both rice matrix and herbicide treatment. The ion at $m/z$ 701 can be associated with phospholipids from the phosphatidic acid class, such as dioleoyl phosphatidic acid. This class of lipids is categorized as membrane lipids and serves as a rich source of signaling molecules in response to various stresses encountered by plants such as rice.[46] Its expression in the CH group was significant for group classification, indicating the stress to which the plant was subjected. Furthermore, putative annotation can relate $m/z$ 381 with the presence of monoacylglycerols. This molecule was detected in the rice husk that may reflect lipid metabolic activity and stress responses, potentially contributing to cuticular properties and defense mechanisms in this protective tissue.[47]

Notably, $m/z$ 393, which showed a high VIP score in the husk (H) matrix in the previous model, now displays a lower VIP score specifically in the herbicide-treated group. This reduction in VIP score for $m/z$ 393 within the treated samples


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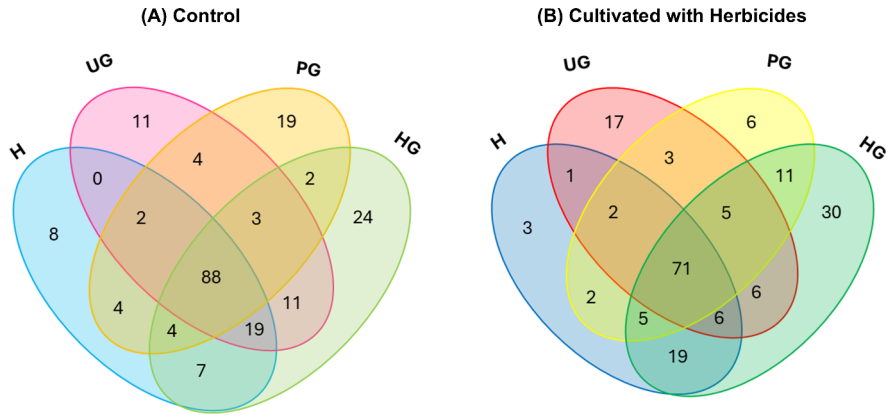


**Figure 6.** Venn diagrams of four distinct matrices: rice husk (H), husk and grain (HG), polished grain (PG), and unpolished grain (UG) for the control samples (A) and herbicide-treated rice samples (B).

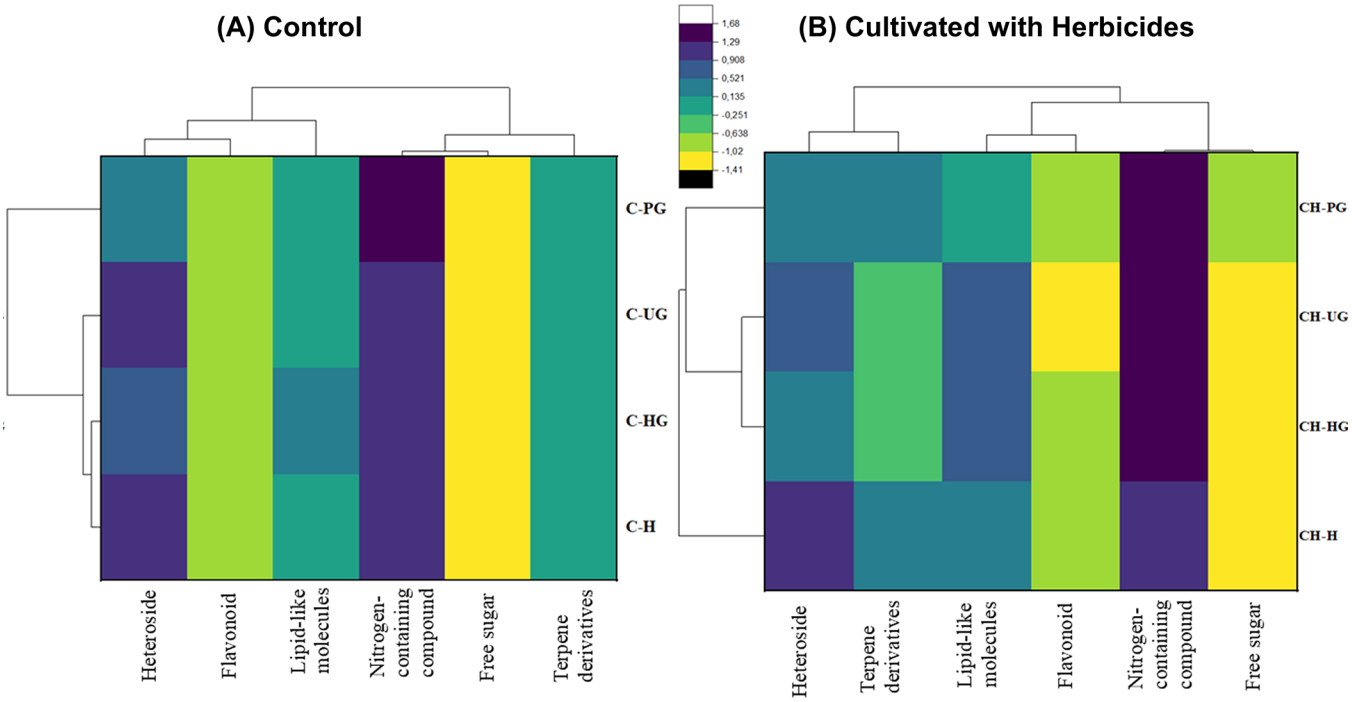


**Figure 7.** Comparative analysis of primary and secondary metabolite classes in rice matrices (husk, husk and grain, polished grain, and unpolished grain) under herbicide-treated and control conditions. The analysis includes the following compound classes: heterosides, lipid-like molecules, nitrogen-containing compounds, free sugars, flavonoids, and terpene derivatives.

suggests that the presence or abundance of this metabolite may have been suppressed or altered by herbicide exposure.[47] Given its initial importance in the husk, which may serve a protective or defensive role, the decreased VIP score in the treated group might indicate that herbicides impact its synthesis or accumulation. This observation highlights the potential influence of herbicides on specific metabolites associated with environmental response, particularly in the protective layers of the rice grain. The $m/z$ 393 ion may be associated with a phosphatidic acid 16:0. These molecules may be present in rice husk and function as intermediate metabolites in the biosynthesis of storage lipids and recognized as signaling molecules that respond to adverse conditions through their accumulation or depletion.[48]

The VIP scores from the PLS-DA models identified several $m/z$ values as the most informative for class separation, with consistent metabolites appearing as key contributors across different models. However, these $m/z$ values likely belong to central metabolic pathways, as they were important in all classes, regardless of herbicide treatment or rice matrix. This observation suggests that the VIP score analysis did not reveal substantial differences in specific metabolites that could be directly associated with herbicide exposure or its varying effects across rice matrices. To gain a deeper understanding of the metabolomic shifts related to herbicide treatment, a putative annotation was subsequently performed.

**3.3. Metabolomic Profile of Rice through HPLC-HRMS Analyses.** An untargeted metabolomic approach was employed to assess the metabolomic changes in rice subjected to herbicide application, as well as the chemical composition changes of rice parts after producer processing. The Venn diagram (Figure 6) was utilized to explore the metabolic variations among analyzed samples and the number of metabolites shared between the matrices, aiming to evaluate


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the effect of herbicides on the production of primary and secondary metabolites in rice. Additionally, the influence of processing on the abundance and presence of metabolites in polished and unpolished grains was evaluated, along with variations in chemical composition between the husk and grain. These factors are critical as they impact the nutritional quality and biological properties of rice.

Initially, compositional differences between the rice parts (husk and grain) and the effect of polishing were investigated. Subsequently, the same matrices were evaluated based on the cultivation conditions (with or without herbicide treatment). The Venn diagram revealed that for the herbicide-treated samples, the husk and the husk-and-grain matrices shared about 80% of their metabolome, while polished and unpolished grains showed approximately 75% overlap. However, the unpolished grain consistently showed a higher number of unique metabolites compared to the polished grain, exhibiting 25% more unique metabolites compared to the polished grain, indicating that processing impacts metabolite diversity.

When comparing all herbicide-treated matrices, they shared a majority of their metabolome, but distinct differences were observed, with the husk and unpolished grain showing more distinct metabolites than the polished grain. The Venn diagram identified 8 unique ions for the husk samples, and 52 unique ions for the husk-and-grain matrix, with 101 ions shared between them. Polished and unpolished grains exhibited 81 common ions, 30 ions unique to unpolished grains and 24 ions unique to polished grains. This suggests that the herbicide treatment affects the metabolome differently depending on the rice part.

Finally, comparing the herbicide-treated samples with the control, it was evident that the husk and husk-and-grain matrices had the highest similarity, sharing a large percentage of their chemical composition. The husk samples share 96 common ions, with 13 ions unique to the herbicide-treated samples and 36 ions unique to the control. However, the unpolished grain displayed 18% more unique metabolites in both conditions, highlighting its distinct metabolic profiling. Polished grain samples were more similar to each other regardless of herbicide treatment, sharing 70% of the metabolome, which indicates that processing reduces the metabolic differences caused by herbicide exposure. These findings highlight the key differences in metabolite profiles across the various conditions and treatments, emphasizing the influence of herbicides, processing, and rice parts on metabolomic profiling.

Additionally, using *in silico* dereplication tools, the abundance of primary and secondary metabolite classes was evaluated (Figure 7) by analyzing the number of annotated compounds in the rice husk (H), husk and grain (HG), polished grain (PG), and unpolished grain (UG) matrices, both with herbicide treatment (cultivated with herbicide - CH) and without (control - C). The study assessed highly polar primary metabolites such as free sugars, less polar molecules like lipid-like compounds, and nitrogen-containing compounds, which include both primary metabolites like amino acids and secondary metabolites like alkaloid derivatives. The most abundant classes of secondary metabolites identified were flavonoids and terpene derivatives. The content of secondary metabolites in molecules containing one or more sugar units (heterosides) was also evaluated, with most of the glycosylated compounds being associated with saponins containing multiple units of rhamnose and glucose.

In the herbicide-treated samples, the husk showed the highest abundance of heterosides among all matrices, while the abundance of free sugars was similar across husk, husk and grain, polished grain, and unpolished grain matrices. The husk and grain matrix had the highest number of nitrogen-containing compounds, surpassing the other matrices, including the husk alone. Flavonoid content was relatively consistent between the husk and husk and grain matrices, but the unpolished grain had approximately 50% fewer available flavonoids. Terpene derivatives were more abundant in the husk when compared to the other rice parts, with the husk and grain matrix showing a moderate amount, and the unpolished grain having the least.

When comparing the control samples, the unpolished grain matrix exhibited the highest abundance of heterosides, with about 20% more than the husk and grain matrices, significantly more than the polished grain. The control husk and grain matrix contained the highest number of nitrogen-containing compounds, similar to what was observed in the herbicide-treated samples, but the control unpolished grain also showed a significant amount. Flavonoid content was slightly higher in the control husk compared to the herbicide-treated husk, with the control husk and grain matrix displaying the highest abundance. Terpene derivatives were more prominent in the control husk and grain matrix compared to the herbicide-treated matrices.

In general, the herbicide-treated samples had lower free sugar content compared to the control samples across all matrices. Additionally, the husk in the herbicide-treated condition had about 15% fewer flavonoids than in the control condition, suggesting that herbicide treatment may reduce flavonoid levels. The unpolished grain in both conditions consistently showed higher levels of nitrogen-containing compounds, indicating a robust metabolic profile regardless of herbicide treatment.

Flavonoids represent one of the most significant classes of polyphenols in rice samples, playing a crucial role in the biological and nutritional properties of the food.[49,50] These compounds can be found either as aglycones or as heterosides containing one or more sugar units. Glycosylated flavonoids in rice were identified by their neutral loss of sugar moieties, with hexosides showing a loss of 162 Da, deoxyhexosides 146 Da, and pentosides 132 Da in *O*-glycosylated flavonoids.[51] C-glycosylated flavonoids were characterized by the loss of $H_2O$ and 120 Da.[51]

The following flavonoids were putatively annotated: acacetin 7-rutinoside, methyquercetin glucoside, diosmetin 7-rutinoside, 5,7,3′-trihydroxy-4′-methoxyflavanone, isoetin 7-glucoside-2′-xyloside, isoswertisin 4′-glucoside, delphinidin 3,5-diglucoside, and luteolin 7-glucuronylglucoside. All the annotated flavonoids were previously reported in rice.[50] The abundance (absolute signal intensity in MS analysis) and presence of these eight flavonoids were compared between herbicide-treated and control samples to determine if cultivation conditions impact the production of this class of metabolites.

In control samples, diosmetin 7-rutinoside and luteolin 7-glucuronylglucoside were abundant, with luteolin 7-glucuronylglucoside being present only in controls and absent in herbicide-treated samples. diosmetin 7-rutinoside was also higher in controls, suggesting reduced levels with herbicide application. Conversely, Acacetin 7-rutinoside and methyquercetin glucoside were unique to herbicide-treated samples, with


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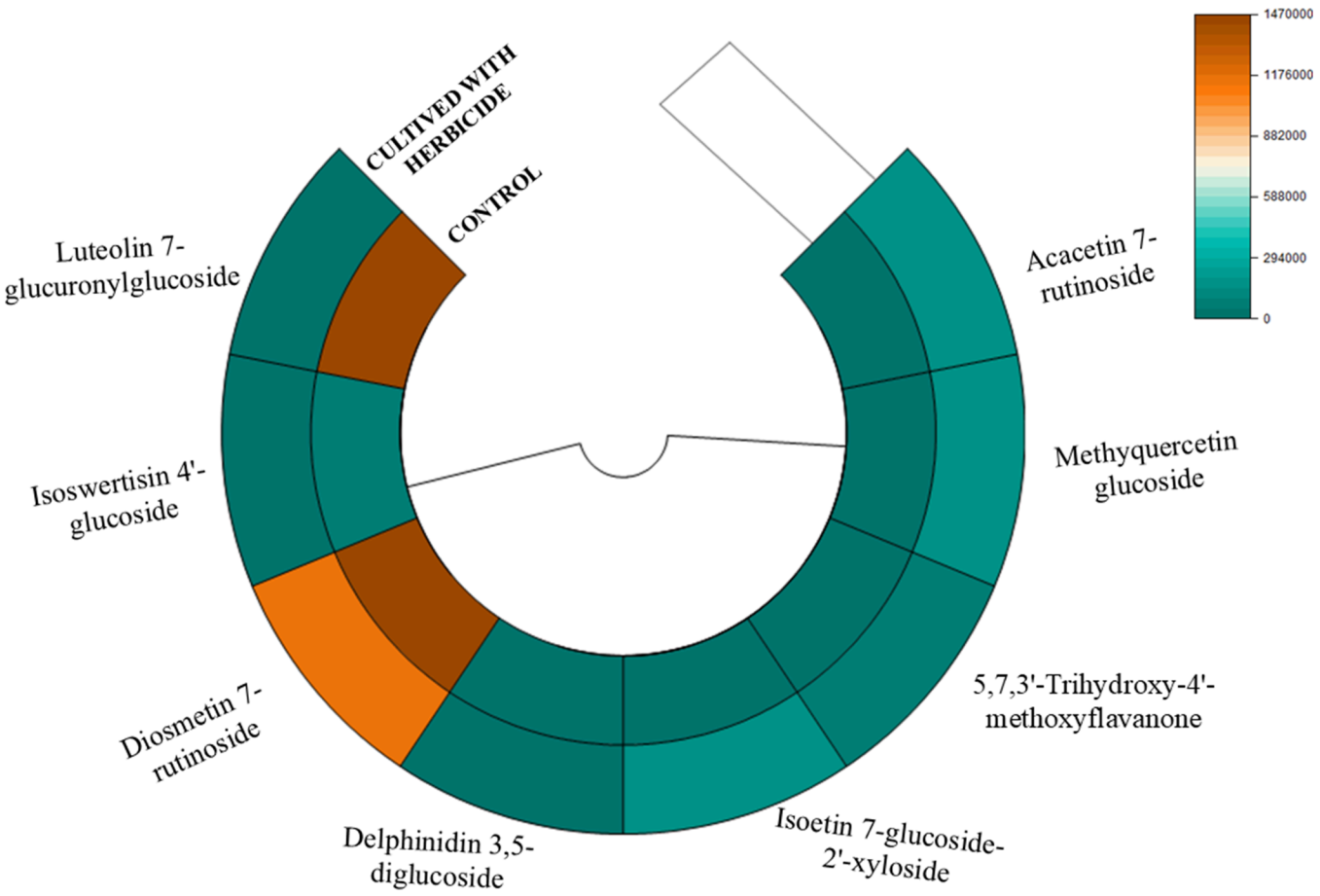


**Figure 8.** Comparison of flavonoid profiles in rice under control and herbicide-treated conditions. The figure highlights the significant impact of herbicide treatment on flavonoid composition, including the flavonoids Acacetin 7-rutinoside, Methyquercetin glucoside, Diosmetin 7-rutinoside, 5,7,3′-Trihydroxy-4'-methoxyflavanone, Isoetin 7-glucoside-2'-xyloside, Isoswertisin 4'-glucoside, Delphinidin 3,5-diglucoside, and Luteolin 7-glucuronylglucoside.

Acacetin 7-rutinoside being notably abundant. Methyquercetin glucoside was significantly higher in herbicide-treated samples, indicating a possible metabolic response to herbicide exposure. Flavonoids like 5,7,3′-trihydroxy-4'-methoxyflavanone and isoetin 7-glucoside-2'-xyloside were found in both conditions but varied in levels. Isoswertisin 4'-glucoside and delphinidin 3,5-diglucoside were less abundant in herbicide-treated samples, with delphinidin 3,5-diglucoside present only in these samples. Overall, herbicide treatment notably alters the flavonoid profile in rice, with some flavonoids unique to each condition and others varying in abundance (Figure 8).

The untargeted metabolomic approach was successfully employed to assess the impact of herbicide application on the production of secondary metabolites in rice. The results revealed metabolites that can be used to differentiate between groups and may be associated with the biochemical response of rice to the herbicide used in cultivation. For instance, the amino acid tryptophan, previously reported in rice, was detected in both control and herbicide-treated conditions. However, its derivative tabersonine ($C_{21}H_{24}N_2O_2$), was only found in control rice husk and grain samples. This finding is consistent with Issa (1999)[52] and Means (2004),[41] who observed partial inhibition of aromatic amino acid synthesis, such as tryptophan, in cyanobacteria due to glyphosate's interference in the shikimic acid pathway. Adenosine was also detected in the samples and has been previously reported as a differential metabolite in the soil, roots, and shoots within the rice production system.[53]

The spectral library search returned numerous hits for glycosylated compounds (heterosides), predominantly glucopyranosyl and rhamnopyranosyl forms. The application of the *in silico* tool Network Annotation Propagation (NAP), which utilizes spectral networks to enhance the ranking of fragmentation candidate structures, revealed several compounds that can differentiate between control and herbicide-treated rice samples. For example, in the herbicide-treated rice group was detected a lactone with $m/z$ 209, flavonoid 3′,6-dimethylflavone ($m/z$ 273 $[M + Na]^+$), two hydroxylated aliphatic compounds (policosanols) with $m/z$ 281 and 353, and a phenylpropanoid with $m/z$ 317. In contrast, the control group contained a lactone with $m/z$ 257 and several policosanols such as heneicosanol, docosanol, and tetracosanol, which were not found in herbicide-treated samples.

Additionally, the mass spectral database search and *in silico* fragmentation tools, such as MolDiscovery and Dereplicator+, identified Myo-inositol exclusively in herbicide-treated samples and Tocotrienol solely in control samples. Saponins were primarily found in herbicide-treated rice samples. A labdadiene rhamnopyranoside derivative ($m/z$ 592 $[M+2H]^+$ 297) was detected only in herbicide-treated samples, while its non-glycosylated derivatives, labdadiene ($m/z$ 321 and 336), were only present in control samples. Furthermore, three galloyl-


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containing compounds ($m/z$ 359, 299, and 301) were exclusively found in herbicide-treated samples.

A comparative analysis between samples with and without fungicide treatment revealed distinct shifts in metabolic pathways and compound classes. Steroidal compounds and nitrogen-containing compounds were predominantly associated with fungicide-treated samples, suggesting the activation of steroid biosynthesis and alkaloid metabolism. In contrast, key secondary pathways such as those derived from tryptophan and phenylpropanoids were notably repressed, as indicated by the absence of their downstream metabolites. Despite the presence of tryptophan in both conditions, its lack of conversion in treated samples points to a metabolic bottleneck. Additionally, an altered diversity of lipids and sugars suggests impairment of structural and energy-related metabolism. Meanwhile, core metabolic routes remained partially active, as evidenced by the detection of shared primary metabolites. These findings highlight the selective reprogramming of rice metabolism under fungicide pressure, with pronounced effects on bioactive secondary pathways, an effect similarly reported in herbicide-treated crops, where the biosynthesis of phenylpropanoids was significantly disrupted.[54]

This study showed that the untargeted metabolomic approach effectively distinguishes rice matrices (polished and unpolished grains, husk, and husk with grain) cultivated with and without herbicide. Herbicide application impacted the secondary metabolite profile, reducing flavonoid levels and altering nitrogen-containing compounds. Unpolished grains displayed a richer metabolite profile, and compounds like tabersonine were found only in samples without herbicide, highlighting the potential of metabolomics to reveal biochemical responses.

This metabolomic analysis revealed significant alterations in the composition of nitrogen-containing compounds and flavonoids between control and herbicide-treated rice samples. Nitrogen-containing metabolites, including amino acids and alkaloid derivatives, were consistently more abundant in the husk and grain matrices, with a marked increase in the unpolished grain, regardless of treatment condition—indicating a robust nitrogen metabolism in these tissues. However, the most notable differences were observed in the flavonoid profiles. Several flavonoids were either exclusively present or significantly more abundant in control samples, suggesting a potential suppression of flavonoid biosynthesis upon herbicide exposure. Conversely, certain flavonoids like acacetin 7-rutinoside and methyquercetin glucoside were unique to herbicide-treated samples, indicating a possible compensatory or stress-induced metabolic response. These findings underscore the impact of herbicide application on secondary metabolism in rice and highlight specific classes of metabolites that may serve as biomarkers of chemical stress.

In summary, these efforts demonstrated that untargeted metabolomics effectively distinguish rice matrices and reveal herbicide-related changes in metabolite profiles, such as variations in flavonoid levels and nitrogen compounds. Thus, this study can significantly contribute to understanding how herbicide application in rice cultivation influences the grain's metabolome and the nutritional composition impacts, paving the way for rational herbicide use and supporting improved rice yields and global food security.

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### Author Contributions

§A.C.B.J., J.V.R., and N.M.L.: equal contributions to this work.


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## ABBREVIATIONS USED

C: control
CH: cultivated with herbicides
FBMN: Feature-Based Molecular Networking
GNPS: Global Natural Products Social Networking
H: rice husk
HG: husk and grain
HPLC: high-performance liquid chromatography
MN: Molecular Networking
HRMS: high-resolution mass spectrometry
NAP: Network Annotation Propagation
PC: principal components
PCA: principal component analysis
PG: polished grain
PLS-DA: partial least squares discriminant analysis
UG: unpolished grain
VIP: variable importance in projection



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