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Unveiling Diversity in Amino Acid Stable Isotope Profiles for Classifying Rice Varieties, Refining Types and Cultivation Systems

Zoe Giannioti^{1,2}, Alberto Roncone¹, Luana Bontempo^{1,*}

¹ Fondazione Edmund Mach, Via E. Mach 1, 38098 San Michele all'Adige, TN, Italy

² Centre for Agriculture, Food and Environment (C3A), University of Trento and Fondazione Edmund Mach Via E. Mach 1, 38098 San Michele all'Adige, TN, Italy

* Corresponding author. Email: luana.bontempo@fmach.it

Abstract

Isotope Ratio Mass Spectrometry (IRMS) is a promising tool in organic authentication cases. Premium-priced Italian rice varieties (Carnaroli, Arborio, Baldo) are used in cuisines worldwide for their unique qualitative properties. Organic authentication of rice by morphological assessment is unfeasible, while its market availability at different refining stages (brown, white) further increases the data variability. In this study, bulk and compound-specific (CS) - IRMS analysis of nine rice amino acids (AAs), by elemental analyser (EA) – IRMS and gas chromatography (GC) - combustion (C) - IRMS, respectively, were applied in order to explore their organic authentication potential in cases involving different rice varieties and refining types. The individual and interactive effects of the different variables were assessed on the $\delta^{13}\text{C}_{\text{AAs}}$, $\delta^{15}\text{N}_{\text{AAs}}$, $\delta^{13}\text{C}_{\text{bulk}}$ and $\delta^{15}\text{N}_{\text{bulk}}$, and the sample classification was attempted by linear discriminant analysis (LDA) and decision tree analysis (DTA). Organic authentication of brown rice was achieved by CS-IRMS. Generic rice was differentiated from all Italian organic and conventional varieties ($\delta^{15}\text{N}_{\text{leucine}} < 2.5 \text{ ‰}$). The $\delta^{13}\text{C}$ values of glutamic acid, glycine, phenylalanine and proline, significantly contributed to the complete LDA separation of conventional Arborio, conventional Carnaroli and organic Carnaroli samples. This study showcases the interplay between refining type, variety and cultivation, which should be considered in cases of organic authentication by IRMS methods.

1. Introduction

Approximately 50% of rice (*Oryza sativa* L.) in the European Union is produced in Italy, predominantly in the Northern regions following the course of river Po, i.e. Piedmont, Lombardy, Veneto and Emilia, thanks to the presence of flat, sumptuous lands and irrigation channels (Riccio, 2022). The most common Italian rice cultivars belong to the japonica variety and their total number in the National Registry exceeds 130. These include Carnaroli, which is elastic, resistant and ideal for risotto dishes, Arborio, with wide grains and the ability to

maintain large amounts of starch when cooking, and Baldo, which has an elongated grain and is the richest variety in minerals (*Riso Delta Po PGI*, 2020). Even though Italian cultivars possess different qualitative properties, such as starch, protein, fiber and lipid content, their morphological differences are minimal and dependent on stage-specific expression and environmental factors (Cirillo et al., 2009). Varieties with high commercial value such as Carnaroli, which is considered one of the most precious genotypes, can be targeted in fraudulent activities of adulteration or substitution with less costly cultivars (Grazina et al., 2022).

The transition towards organic agriculture in the Italian rice sector is a prominent topic (Vaglia et al., 2022), since rice crops are heavily dependent on the use of agrochemicals, resulting to significant water quality degradation in the Northern Italian areas. Surveys involving Italian consumers have confirmed their willingness to pay premium prices for organic products (Perrini et al., 2010), perceiving them as healthier, safer and richer in nutrients than conventional foods. However, it is not possible to morphologically assess the authenticity of organic products sold in the market. Furthermore, the availability of market choice between brown and white rice can have further implications on the identification of markers for organic rice authenticity. Brown rice includes the endosperm, embryo and bran layers and is superior to white rice as a source of vitamins, minerals, fibers and proteins, as these are unevenly distributed in the kernel, and are lost during the milling process (Mir et al., 2020).

Studies employing isotope ratio mass spectrometry (IRMS), have examined the individual effects of refining type, variety and cultivation on the stable isotope values of different food products. The distinct cultivation practices applied in organic and conventional agriculture directly affect the plant stable isotope profiles, due to the different fertilizer isotope values and the fertilizer effect on the plant respiration rates (Giannioti et al., 2024). Discrimination between organic and conventional milled rice has been achieved based on the bulk $\delta^{13}\text{C}$ (Chi et al., 2024) and $\delta^{15}\text{N}$ values (Chi et al., 2024; Chung et al., 2021). Moreover, significant cultivar differences were observed in the $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ values of organic and conventional oranges, peaches and strawberries, with $\delta^{15}\text{N}$ appeared to be less influenced (Camin et al., 2011). Studies on rice have reported contradictory results. Chung et al. (2016) reported no varietal effects on the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of Korean brown rice, while other studies noted that both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values exhibited differences among rice varieties (Liu et al., 2020) (Chen et al., 2016). Lower bulk $\delta^{15}\text{N}$ values in some varieties of organic rice (*Oryza sativa* L. Japonica), were potentially attributed to more rapid uptake of nutrients taking place prior to denitrification (Yuan et al., 2018). Lastly, differences in the refining stages of rice were observed between milled and brown rice in Korea, based on their bulk $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$

values (Chi et al., 2024), while the $\delta^{13}\text{C}$ values of wheat bran were found to be significantly lower than those of other fractions (Wadood et al., 2018).

Compound-specific (CS) IRMS methods can prove more effective than bulk in the discrimination of organic and conventional food products, providing information on individual components (e.g. amino acids, fatty acids, nitrate) by the addition of a separation step prior to the isotope analysis (Kelly et al., 2018). In this work, bulk and CS amino acids analysis was applied by Elemental Analyser (EA) - IRMS and Gas Chromatography (GC) - Combustion (C) - IRMS, respectively. The aim was to obtain the stable isotope profile of different Italian rice varieties (Carnaroli, Arborio, Baldo, S. Andrea, Rosa Marchetti), refining types (brown/white) and cultivations (organic/conventional). All samples in this study were collected in Northern Italy, in order to ensure no effect of geographic origin variability on the isotope values of different varieties (Perez et al., 2006). The findings of this research aid in the identification of promising stable isotope markers for the organic authentication of cereals.

2. Materials & Methods

2.1 Sample Collection

Eighteen authentic organic rice samples harvested in 2022 were collected directly from certified organic producers in Northern Italy (Pavia and Vercelli provinces in the regions of Lombardy and Piedmont, respectively). Additionally, two organic and eighteen conventional samples were bought from local supermarkets in Northern Italy. All rice samples were medium- and long- grain Italian varieties, including Carnaroli, Arborio, Baldo, Rosa Marchetti and S. Andrea. The complete sampling details can be seen in Table A1.

2.2 Bulk Analysis

Samples were weighed (ca. 2 mg) and placed in tin capsules to measure the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values simultaneously, using an isotope ratio mass spectrometer (Isoprime visION, Elementar Analysensysteme, Langensfeld, Germany) after total combustion in an elemental analyser (Vario Isotope Cube, Elementar Analysensysteme, Langensfeld, Germany). All samples were measured in triplicate.

The isotope ratios were expressed in δ ‰ versus atmospheric nitrogen for $\delta^{15}\text{N}$, and V-PDB (Vienna – Pee Dee Belemnite) for $\delta^{13}\text{C}$, according to the equation below, where R is the ratio of the heavy (^iE) to light (^jE) isotope of an element E:

$$\delta^i(E_{\text{sample/standard}}) = \frac{R(^i\text{E}/^j\text{E})_{\text{sample}}}{R(^i\text{E}/^j\text{E})_{\text{standard}}} - 1$$

International reference materials (U.S. Geological Survey), and an in-house working standard (wheat flour), were used to normalise the isotopic values, namely, USGS90 (millet flour, $\delta^{15}\text{N}$: 8.84 ‰, $\delta^{13}\text{C}$: -13.75 ‰) and USGS88 (collagen, $\delta^{15}\text{N}$: 14.96 ‰, $\delta^{13}\text{C}$: -16.06 ‰).

2.3 CS Amino Acids Analysis

2.3.1. Reagents and materials

L-Amino acid standards at $\geq 98\%$ purity (alanine, aspartic acid, glutamic acid, glycine, isoleucine, norleucine, leucine, phenylalanine, proline, and valine) and analytical grade cation-exchange resin (Amberlite IR120 hydrogen form) were purchased from Sigma-Aldrich. All other solvents (isopropanol, acetone, and ethyl acetate) and reagents (triethylamine and acetic anhydride) used were of analytical grade and purchased from Sigma-Aldrich and VWR (Milan, Italy).

2.3.2 Sample preparation and analysis

A multi-step sample preparation process was followed for the GC-C-IRMS analysis of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ wheat amino acids, as described by Paolini et al. (2015). This involved defatting the samples with a mixture of petroleum ether/ethyl ether, followed by protein hydrolysis with HCl, and amino acid purification using an ion-exchange chromatography resin. N-acetyl isopropyl derivatization was the final phase, which required acidified isopropanol for esterification and a mixture of acetic anhydride/trimethylamine/acetone for acetylation.

The isotopic values of 8 amino acids, alanine (Ala), aspartate (Asx), glutamate (Glx), glycine (Gly), leucine (Leu), phenylalanine (Phe), proline (Pro), threonine (Thr), and valine (Val), were determined by a Trace GC Ultra (GC IsoLink + ConFlo IV, Thermo Scientific) interfaced with an IRMS (DELTA V, Thermo Scientific) through an open split interface and with

a single-quadrupole GC-MS (ISQ Thermo Scientific). Due to the conversion of asparagine (Asn) and glutamine (Gln) into aspartate (or aspartic acid) (Asp) and glutamate (or glutamic acid) (Glu), after the acid-hydrolysis step, the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ reported in the samples represent their summaries as Asx and Glx.

All samples were measured in duplicate. Corrections accounting for the measured $\delta^{13}\text{C}$ values of the derivatized amino acids were carried out as reported in Paolini et al. (2015).

2.4 Statistical analysis

All statistical analyses were carried out using R (version 4.4.0).

Multivariate Analysis of Variance (MANOVA) was used to investigate whether the type of rice (brown or white), variety and cultivation (organic or conventional), or their interaction, had an effect on the stable isotope values of the amino acids analysed in this study. A correlogram was used to assess the randomness of the dataset.

Linear Discriminant Analysis (LDA) was carried out in order to separate the classes based on variety and cultivation method (conventional/ organic), regardless of the type (white, brown). The classes considered were the ones with sample number ≥ 5 . The numerical variables included all $\delta^{15}\text{N}_{\text{AAs}}$, $\delta^{13}\text{C}_{\text{AAs}}$, $\delta^{15}\text{N}_{\text{bulk}}$ and $\delta^{13}\text{C}_{\text{bulk}}$. Prior to fitting an LDA model, class weights were defined according to the number of instances in each class, the numerical variables were standardized and the data was split into Training and Test sets (80:20 split) using stratified sampling to maintain the proportion of each class in the split. Subsequently, predictions were made using the LDA model on the test set, ensuring that predicted and actual values were factors with the same levels. The model performance was evaluated using a confusion matrix and a 3D scatter plot was created for the visualization of the LDA scores and the class separation.

Finally, a decision tree was constructed to aid in the decision-making process of classifying rice variety and cultivation. The steps included standardization of the numerical variables, stratified sampling while performing an 80:20 split of the data into Training and Test sets, fitting the decision tree model, predicting on testing data and ensuring that the predicted and actual values were factors of the same level, before plotting the decision tree. The pruning parameter was 0.01 (default), the minimum number of observations for a node to split was 10, the minimum number of observations in any leaf was 3, and the maximum depth of any node was set to 5 (moderate).

3. Results & Discussion

3.1 Rice Variety, Refining Type and Cultivation Effects

The individual and combined effects of refining type, cultivation and variety on the amino acid and bulk stable C and N isotope values were initially examined and are shown in Table 1. Significant influence of the three different factors individually was observed in the amino acid $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ profile of the samples, with different effects seen for each amino acid separately. Alanine $\delta^{15}\text{N}$ values were the most unaffected $\delta^{15}\text{N}_{\text{AAs}}$ by all factors, while glutamic acid $\delta^{15}\text{N}$ was the most affected. On the other hand, valine and isoleucine $\delta^{13}\text{C}$ values were the only $\delta^{13}\text{C}_{\text{AA}}$ significantly affected. The interaction assessment showcases that the influence of Type on the AA $\delta^{13}\text{C}$ profiles varies depending on the cultivation, and vice-versa. Similarly, but to a lesser extent, the influence of Type on the AA $\delta^{15}\text{N}$ profiles varies according to the specific Variety of rice, and vice-versa.

Table 1. MANOVA results for the interactive and individual effects between type, cultivation and variety on the C and N stable isotope values of AAs. Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '.' 1.

	ala		val		ile		leu		gly		pro		asx		glx		phe		bulk	
	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N
Type			*	.			***	.	*	*	***	***	***	***						.
Cultivation			*		*	**	.	*			*	**	***	***	.		*	*		
Variety					*		***		***			**	***	***	**		**	***		.
Type:Cultivation																				
Type:Variety					.		.													

The assessment of the effect of milling on the bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values has shown contradictory results in studies carried out in brown and white (milled) rice in South Korea (Chi et al., 2024; Yun et al., 2011). On the other hand, it has been documented that protein content decreases with increasing milling percentage (Liu et al., 2017), potentially affecting the amino acid stable isotope values. The effect of cultivation on the latter mainly derives from the

different fertilization methods of organic and conventional agriculture, allowing for the discrimination of organic from conventional milled rice based on amino acid $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Chung et al., 2019).

Significant differences were noted between the bulk $\delta^{13}\text{C}$ of japonica and indica varieties in China (Wang&Chen et al., 2020), and between the bulk $\delta^{13}\text{C}$ values of superior and inferior Chinese rice grains, which were attributed to differences in soluble carbohydrate accumulation and starch biosynthesis (Chen & Zhao et al., 2016). Moreover, significant difference was reported in both the bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between basmati and non-basmati cultivars, which were explained by the cultivar genetic variations and, specifically, the diffusive conductance, stomatal activity and water use efficiency (Wadood & Chunlin et al., 2024). Lastly, significant differences were reported between both the protein and amylose content of different Italian rice varieties, including Arborio, S. Andrea and Carnaroli rice (Haxhari et al., 2023), which could explain the results of this study on the significance of cultivar as a variable in amino acid CS-IRMS studies.

The correlations revealed between the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of rice AAs are shown in Figure 1. The lowest coefficients were observed for correlations between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, due to the different metabolic pathways associated with their fractionation (such as the plant photosynthetic pathway for $\delta^{13}\text{C}$ or the nitrogen fixation and uptake for $\delta^{15}\text{N}$).

The amino acids with the highest $\delta^{15}\text{N}$ correlations, i.e. $\delta^{15}\text{N}_{\text{asx}}$ with $\delta^{15}\text{N}_{\text{glx}}$, $\delta^{15}\text{N}_{\text{leu}}$ with $\delta^{15}\text{N}_{\text{asx}}$ and $\delta^{15}\text{N}_{\text{leu}}$ with $\delta^{15}\text{N}_{\text{glx}}$, also exhibited the highest $\delta^{13}\text{C}$ correlations, with the addition of $\delta^{13}\text{C}_{\text{ala}}$ with $\delta^{13}\text{C}_{\text{glx}}$ and $\delta^{13}\text{C}_{\text{asx}}$, and of $\delta^{13}\text{C}_{\text{val}}$ with $\delta^{13}\text{C}_{\text{leu}}$. The high correlations of most $\delta^{15}\text{N}_{\text{AAs}}$ with $\delta^{15}\text{N}_{\text{glx}}$ and $\delta^{15}\text{N}_{\text{asx}}$ can be explained by the amide-N of Gln being the primary nitrogen donor for the synthesis of the other AAs and the amide-N of asn especially contributing to nitrogen storage and transport (Styring et al., 2014). Furthermore, the significant correlations between $\delta^{13}\text{C}_{\text{AAs}}$ can be attributed to all correlated amino acids deriving from intermediates, such as pyruvate (ala, leu, val), α -ketoglutarate (glx) and oxaloacetate (asx), of central carbon metabolic pathways such as glycolysis and the TCA cycle (Lynch et al., 2016).

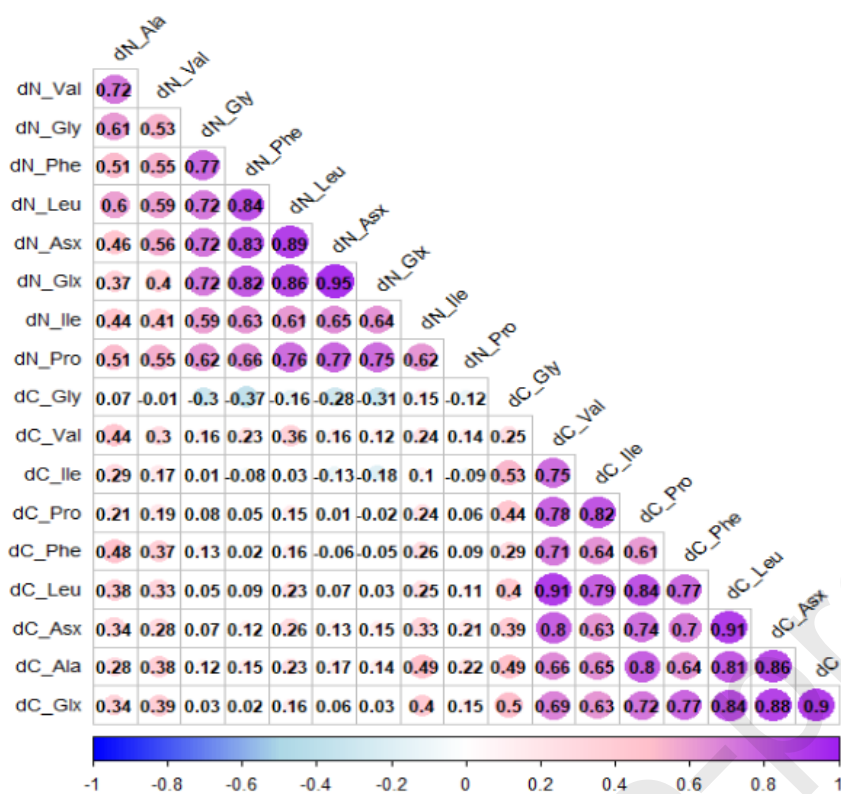


Figure 1. Correlations between the stable isotope ratios of rice AAs.

3.2 $\delta^{13}\text{C}$ Variability Across Type, Variety and Cultivation

The $\delta^{13}\text{C}_{\text{AAs}}$ results are summarized in Figure 2. Unpaired student's t tests revealed statistically significant differences ($p < 0.05$) between the bulk $\delta^{13}\text{C}$ values of the following varieties: Arborio and Carnaroli (white, conventional), Baldo and Rosa Marchetti (white, organic), Baldo and Carnaroli (brown, organic) (Figure A1). Interestingly, none of the $\delta^{13}\text{C}_{\text{AAs}}$ were found significantly different between these combinations, which suggests that rice components different than AAs are what majorly contributes to the variability in the bulk values (such as starch, fatty acids or fibers). The $\delta^{13}\text{C}_{\text{phe}}$ was the only $\delta^{13}\text{C}_{\text{AA}}$ for which a separation of varieties was seen, specifically between Rosa Marchetti and Baldo, however a bigger sample size would be necessary to determine the reliability of this result.

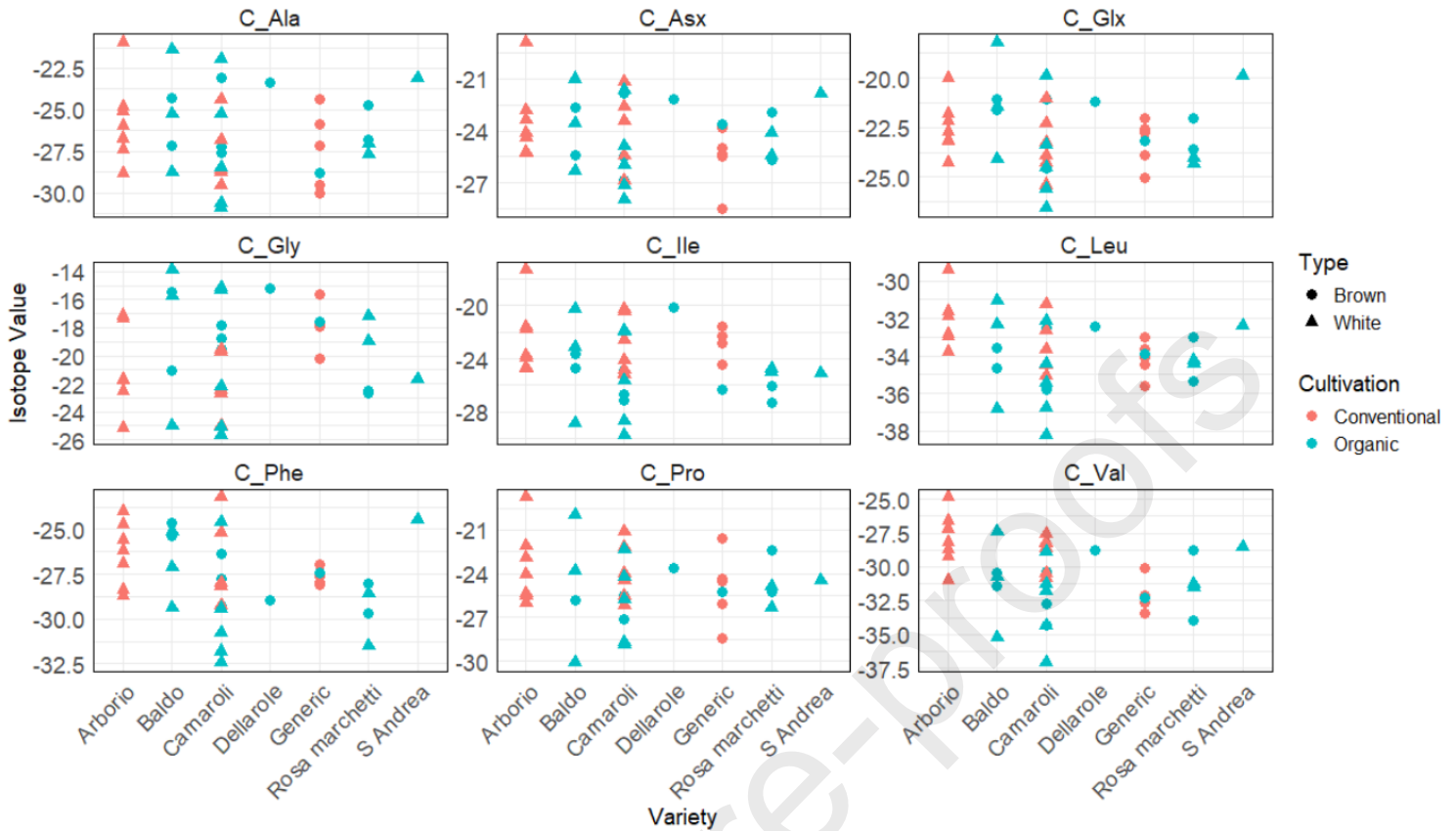


Figure 2. $\delta^{13}\text{C}_{\text{AAs}}$ variability across Italian rice varieties: Arborio (n=7), Baldo (n=5), Carnaroli (n=13), Dellarole (n=1), Generic (n=6), Rosa Marchetti (n=4), S. Andrea (n=1).

The small effect of variety on $\delta^{13}\text{C}_{\text{AAs}}$, and its insignificant influence on type (Table 1), allows for the grouping together of the different varieties according to their type, whilst still keeping separate the two cultivation modes, given their significant effect on the former (Table 1). Brown rice was found to exhibit significantly lower $\delta^{13}\text{C}_{\text{Val}}$ and $\delta^{13}\text{C}_{\text{Ileu}}$ values, but higher $\delta^{13}\text{C}_{\text{Gly}}$ and $\delta^{13}\text{C}_{\text{Phe}}$ values overall in the conventional samples ($p < 0.05$), while no significant differences were observed between the brown and white samples of organic cultivation ($p > 0.05$). Interestingly, bulk $\delta^{13}\text{C}$ values were not found to be significantly different between brown and white rice in neither organic nor conventional rice samples, which is in agreement with previous studies (Chi et al., 2024), exhibiting ranges that varied between -27.55 to -24.89 ‰ and -28.07 to -26.09 ‰ (for organic and conventional samples respectively). This highlights the added value of the extractable information from compound-specific IRMS analysis.

Upon grouping together the varieties (white type), organic samples exhibited lower bulk $\delta^{13}\text{C}$ values (-27.12 ± 0.4 ‰) than the conventional samples (-26.45 ± 0.9 ‰) ($p < 0.05$). In literature, the differences reported between $\delta^{13}\text{C}_{\text{bulk}}$ of the two cultivation types have been < 1 ‰ (Chung et al., 2021), which can be expected since the major factor influencing the $\delta^{13}\text{C}$ values in plants is their photosynthetic pathway, with C3 plants (including rice) exhibiting

values between -21 and -35 ‰ (Badeck et al., 2005). However, the slightly more negative $\delta^{13}\text{C}_{\text{bulk}}$ values found in the organic samples were attributed to the depleted CO_2 released from the decomposition of manures in the rice paddy fields (Yuan et al., 2018). As expected, the differentiation between organic and conventional brown rice samples was only achieved based on the $\delta^{13}\text{C}_{\text{ile}}$ values (-25.78 ± 1.8 ‰ for the former and -23.50 ± 2.4 ‰ for the latter). $\delta^{13}\text{C}_{\text{ile}}$ values were also able to distinguish between white organic and conventional rice in the study of Chung et al. (2019).

3.3 $\delta^{15}\text{N}_{\text{AAs}}$ for Organic Authentication

Due to the significant interaction between type and cultivation for the majority of $\delta^{15}\text{N}_{\text{AAs}}$, as discussed in 3.1, the results in this section were examined separately for the brown and white rice samples. On the other hand, the varieties were grouped together, since no significant interaction was found between variety and cultivation.

Statistically significant separation ($p < 0.05$) was achieved between the brown organic and brown conventional samples based on the $\delta^{15}\text{N}$ values of ala, val, ile, leu, gly, pro, asx, glx and phe (Figure 3). The largest mean difference between organic and conventional brown rice was +5.0 ‰ observed for the $\delta^{15}\text{N}_{\text{glx}}$ values, followed by phe and ile (+4.0 ‰). Notably, these were significantly higher than the difference between the bulk values. However, in the case of the white rice samples, discrimination between the two cultivation systems was only achieved based on $\delta^{15}\text{N}_{\text{ala}}$, with the mean value being 6.9 for the organic and 7.8 for the conventional samples ($p < 0.05$) (Figure A2).

Earlier studies reported similar results for the discrimination between organic and conventional samples based on $\delta^{15}\text{N}_{\text{AAs}}$, without the separation of white and brown samples, indicating $\delta^{15}\text{N}_{\text{glx}}$ and $\delta^{15}\text{N}_{\text{ile}}$, among others, for rice samples (Chung et al., 2019), and $\delta^{15}\text{N}_{\text{ala}}$, $\delta^{15}\text{N}_{\text{phe}}$, $\delta^{15}\text{N}_{\text{ile}}$ for wheat, with differences of over +5.0 ‰ (Paolini et al., 2015). The significantly lower $\delta^{15}\text{N}$ values noted for conventional brown rice compared to conventional white rice can be attributed to the prominent effect of the synthetic fertilizer in the nitrogen isotopic composition of the rice grain, especially in the outer layer, which is retained to a greater extent in the case of the former rather than the latter. On the other hand, organic brown and white rice exhibited more similar ranges, since the $\delta^{15}\text{N}$ profile of organic fertilizers is closer to the natural background levels, resulting in some degree of homogeneity throughout the grain. As explained elsewhere (Choi et al., 2017), the $\delta^{15}\text{N}$ values of synthetic fertilizers (-0.3 ± 0.2 ‰) are significantly lower than those of organic fertilizers such as manure ($+7.8 \pm 0.6$ ‰) and compost ($+16.3 \pm 0.8$ ‰), which is reflected in the $\delta^{15}\text{N}$ of agricultural products.

However, the mean $\delta^{15}\text{N}_{\text{bulk}}$ value of white organic rice (4.5 ‰) was significantly lower than that of white conventional rice (5.5 ‰), while brown rice exhibited similar values for both cultivations (c. 4.3 ‰ for conventional and 4.6 ‰ for organic samples). Several of the organic samples analysed in this study were cultivated on soil where leguminous crops were previously grown. Leguminous plants are N_2 -fixing and exhibit $\delta^{15}\text{N}$ values close to those of atmospheric nitrogen (0 ‰) (Bedard-Haughn et al., 2003), thus lowering the $\delta^{15}\text{N}$ values of the organic rice crops.

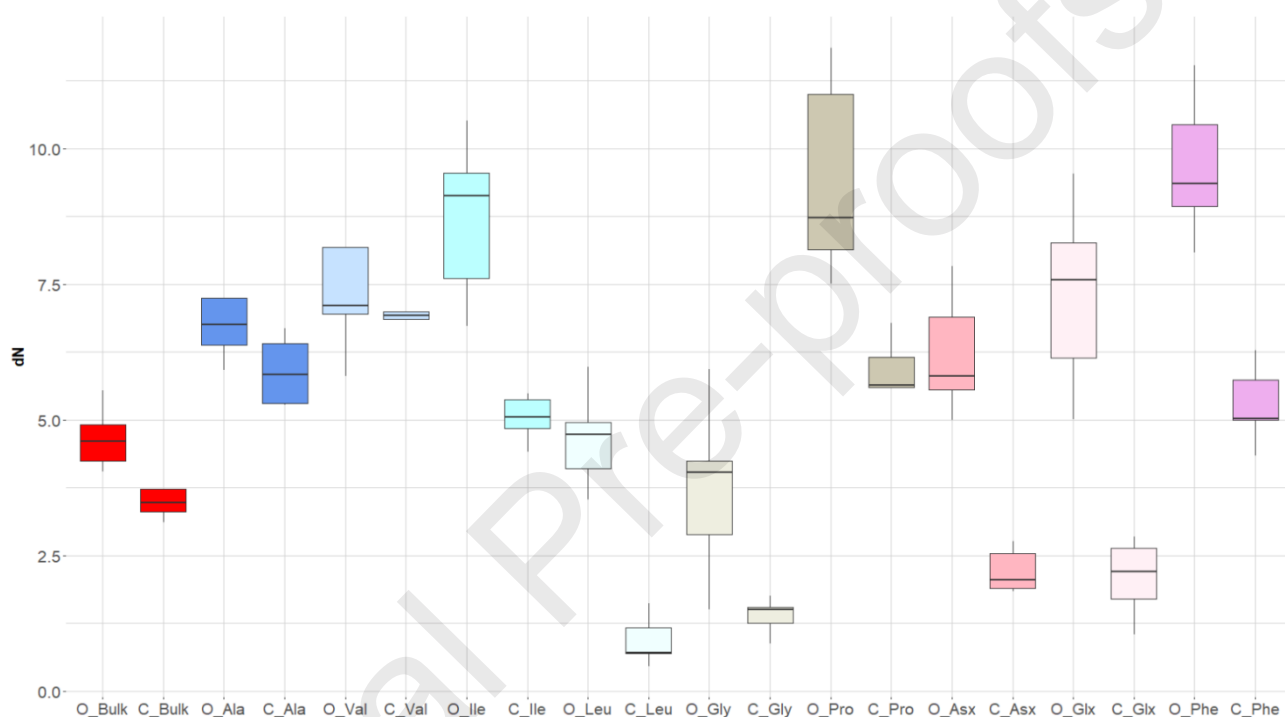


Figure 3. Brown rice $\delta^{15}\text{N}_{\text{AA}}$ (‰) values of organic (O) and conventional (C) cultivation. $p < 0.05$ between the O and C $\delta^{15}\text{N}$ values of ala, val, ile, leu, gly, pro, asx, glx and phe.

3.4 Classification of Rice Variety and Cultivation

3.4.1 Linear Discriminant Analysis (LDA)

The rice samples were classified according to their variety and cultivation in an LDA approach, so as to explore the potential of IRMS in organic rice authentication when different varieties are considered. Type (brown or white) was not taken into consideration in this case since it can be easily assessed by visual inspection.

The confusion matrix and results are shown in Table A2. The model overall correctly classified 80% of the samples, which is satisfactory considering the limited sample size and large variability, with the Kappa coefficient (0.75) showing substantial agreement between raters (Landis&Koch, 1977), and the $p < 0.05$ indicating that the model is significantly better

than the No Information Rate (NIR). Notably, the three major performance metrics of the predictive classification model (precision, recall and specificity), as well as the Receiver Operating Characteristic (ROC) curves (Figure A3), indicated that the model performance was excellent for the classes Arborio_Conv (conventional), Carnaroli_Conv (conventional) and Generic_OrgConv (organic and conventional) (AUC = 1). Moderate number of false positives and negatives was reported for the Carnaroli_Org (organic) class, which still exhibited perfect recall (= 1) and a good ability to distinguish this class from the others (AUC = 0.75). Lastly, the case of Baldo_Org (organic) was more complex, with the model exhibiting low precision, recall and sensitivity, but great specificity, AUC = 1, and clear separation in the 3D space (Figure 4). The low metrics are likely due to the small sample size rather than a limitation of the model. A brief explanation for the terminology of the model interpretation is included in Table A3.

The greatest proportion of Variance was explained by LD1 (c. 85%), followed by LD2 (c. 0.1%) and finally LD3 and LD4 (both below 0.04%). The contribution of each variable to the LDs is depicted in Figure 5. The variables with the strongest positive contribution to LD1, and thus aiding in the separation of the classes Baldo_Org, Carnaroli_Org and Generic_OrgConv, were $\delta^{13}\text{C}_{\text{glx}}$ and $\delta^{13}\text{C}_{\text{gly}}$ (>6), while $\delta^{13}\text{C}_{\text{phe}}$ and $\delta^{13}\text{C}_{\text{pro}}$ exhibited the most negative contribution to LD1 (<-5), followed by $\delta^{15}\text{N}_{\text{leu}}$, $\delta^{13}\text{C}_{\text{leu}}$ and $\delta^{15}\text{N}_{\text{pro}}$ (<-4), thus aiding in the classification of Arborio_Conv and Carnaroli_Conv. The strongest positive contribution to LD2 was exclusively exhibited by $\delta^{13}\text{C}_{\text{leu}}$ (c. 8), separating Arborio_Conv, Carnaroli_Org and Generic_OrgConv, while the highest negative contribution to LD2 was noted by $\delta^{13}\text{C}_{\text{glx}}$ (c. -8) and $\delta^{15}\text{N}_{\text{leu}}$ (c. -6), aiding in the classification of Carnaroli_Conv. The positive coefficients indicate that higher values of these variables result in higher scores on the corresponding LDs, while negative values imply that higher values of these variables result in lower LD scores. Lastly, the greatest absolute variable contribution to LD3 was from $\delta^{15}\text{N}_{\text{asx}}$, which also exhibited the highest absolute coefficient of all other variables and LDs (c. 9), while $\delta^{15}\text{N}_{\text{leu}}$ and $\delta^{13}\text{C}_{\text{glx}}$ contributed the most to LD4 (c. 3).

It can be concluded that the LDA model successfully separated the conventional from the organic Carnaroli rice, as well as the conventional Arborio from both the conventional and the organic Carnaroli samples. Generic rice was also clearly differentiated from all other classes. It is worth noting that $\delta^{13}\text{C}_{\text{AAs}}$ and $\delta^{15}\text{N}_{\text{AAs}}$ contributed significantly more to the LDs compared to the bulk values.

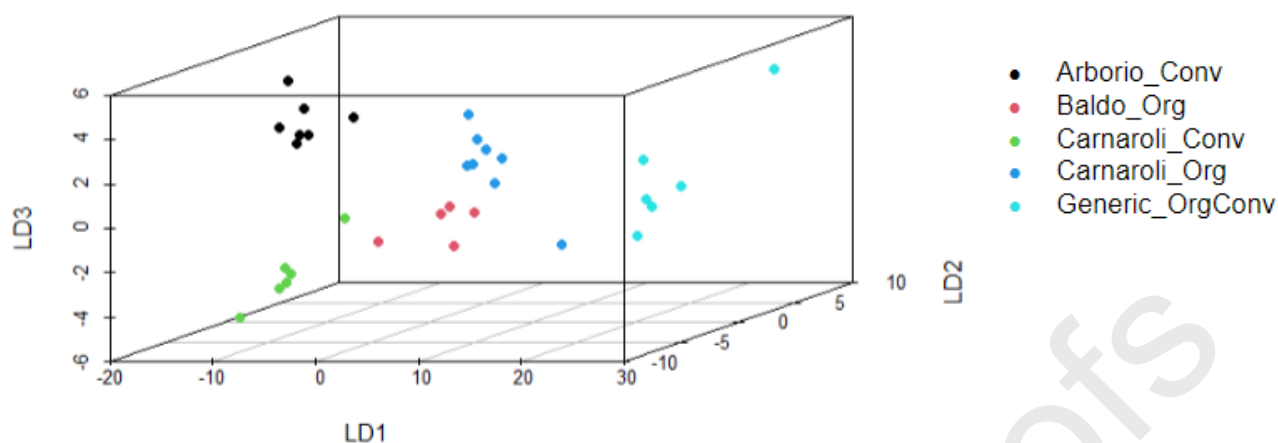


Figure 4. LDA 3D Scatter Plot of LD1 vs LD2 vs LD3 scores for the different classes of rice. Org: Organic. Conv: Conventional.

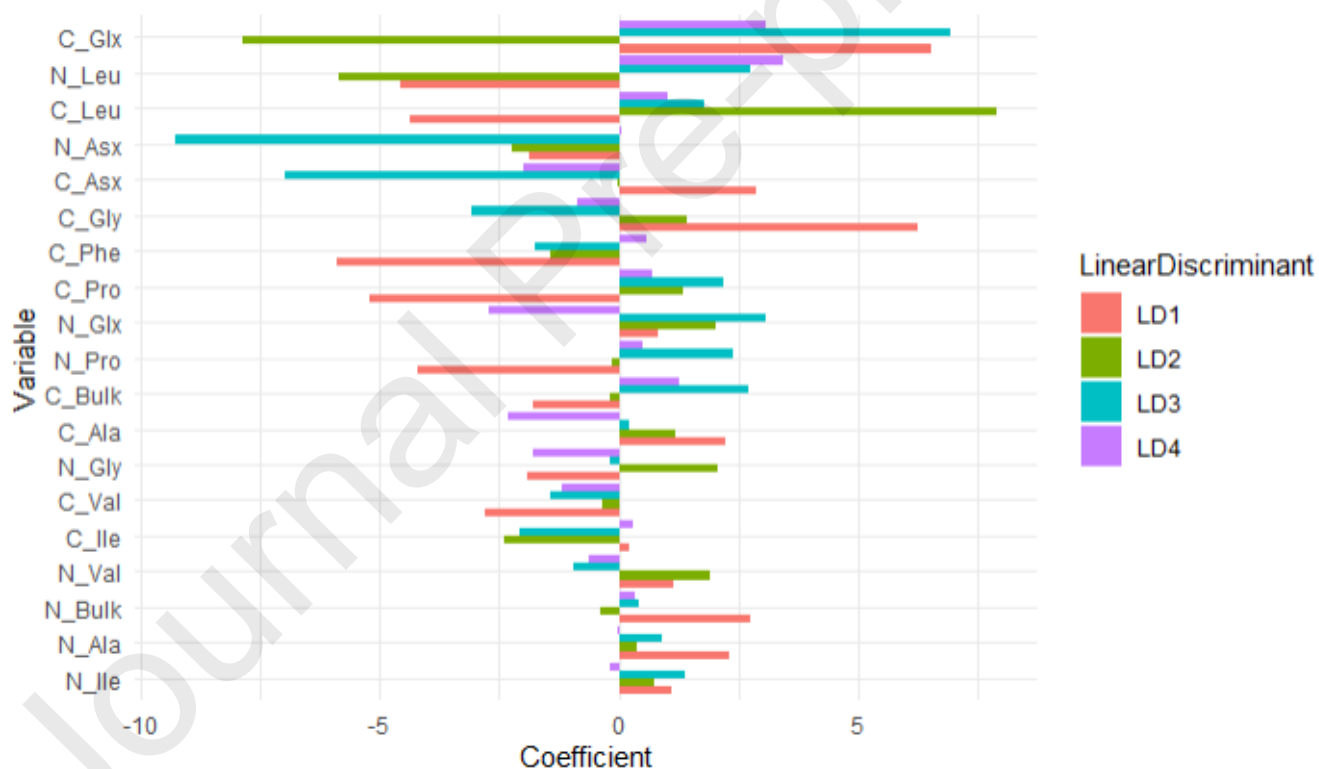


Figure 5. Coefficients of Variables (bulk and AA $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values) for each Linear Discriminant.

3.4.2 Decision Tree Analysis (DTA)

The most important variables indicated by the LDA model, exhibiting absolute

coefficients ≥ 3 , were used to construct a decision tree. The confusion matrix and results are shown in Table A4. Similarly to the LDA results, Arborio_Conv and Generic_Conv exhibited perfect metrics (sensitivity, specificity, balanced accuracy and precision of 1), while better performance was achieved for Carnaroli_Org compared to Carnaroli_Conv, with some false positives detected in the latter, which was the opposite of what was noted in the LDA. Baldo_Org exhibited the least satisfactory performance, an issue which derives from its small sample size, as well as its overlapping features with the Carnaroli_Conv class.

As shown in Figure 6, Generic rice could be differentiated from all other classes based solely on its $\delta^{15}\text{N}_{\text{leu}}$ value, which was lower than 2.5 ‰, with a probability of 1. Additional information derived from the decision tree, is that the $\delta^{13}\text{C}_{\text{bulk}}$ value proved to be a key differentiator between classes, with a value higher than or equal to -26 ‰ indicating Arborio_Conv, and a value between -27 and -26 ‰ indicating Carnaroli_Org, with a probability of 1, when the $\delta^{15}\text{N}_{\text{leu}}$ value was higher than or equal to 2.5 ‰. Moreover, values lower than $\delta^{13}\text{C}_{\text{bulk}}$ -27 ‰ could indicate Carnaroli_Conv or Baldo_Org, depending on the value of $\delta^{13}\text{C}_{\text{val}}$, with some overlap noted between the two classes resulting in reduced probabilities.

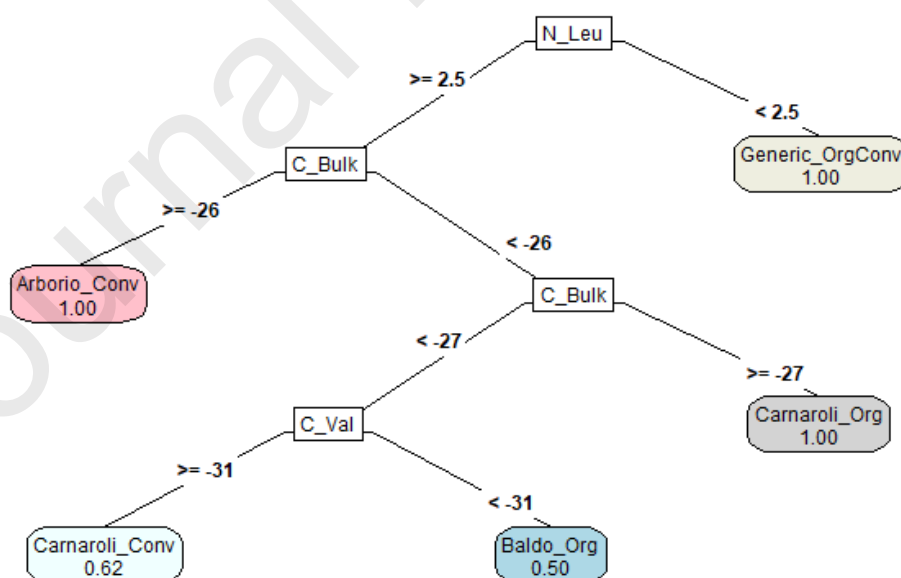


Figure 6. Decision Tree

Conclusions

The variables examined in this study (rice variety, refining type and cultivation) were found to exhibit significant effects on the bulk and AAs $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, both individually and collectively. The effects were more pronounced on the bulk and $\delta^{15}\text{N}_{\text{AAs}}$ values, while $\delta^{13}\text{C}_{\text{AAs}}$ remained less influenced. Understanding these noticeable variations in the stable isotope values reduces the complexity of the data, providing a more in-depth understanding of the implications of bulk and CS-IRMS analysis in organic authentication cases.

Considering exclusively the $\delta^{15}\text{N}_{\text{AAs}}$, organic authentication was only possible for the brown rice samples. However, the addition of $\delta^{13}\text{C}_{\text{AAs}}$ and the results of bulk IRMS combined in an LDA classification, rendered possible the class separation not only of different cultivation systems (organic from conventional), but also of different rice varieties (Arborio from Carnaroli), regardless of refining type. The final application of decision tree analysis aided in the specification of thresholds that could identify the authentic Italian varieties, and separate them from the generic rice, at probabilities reaching 100%.

The novel findings of this study suggest a possibility to apply CS-IRMS techniques, specifically AAs analysis by GC-C-IRMS, in the simultaneous authentication of organic cultivation and plant variety. In order to test the robustness of the method, a bigger number of replicates per class would be needed, as it would also allow for more complex multivariate analysis and potentially the separation of a bigger number of varieties. It would also be interesting to assess the class separation between varieties and cultivations in different sample types, potentially expanding beyond cereals to fruit/vegetables and other plant species.

Acknowledgements

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APPENDIX

Table A1. Italian rice samples list.

No	Cultivation	Variety	Type	Year	Origin (Province)
1	Organic	Rosa Marchetti	White	2022	Rovasenda (VC)
2	Organic	Dellarole	Brown	2022	Rovasenda (VC)
3	Organic	Carnaroli	White	2022	Rovasenda (VC)

4	Organic	Baldo	White	2022	Buronzo (VC)
5	Organic	Baldo	White	2022	Buronzo (VC)
6	Organic	Carnaroli	White	2022	Buronzo (VC)
7	Organic	Carnaroli	Brown	2022	Buronzo (VC)
8	Organic	Baldo	Brown	2022	Buronzo (VC)
9	Organic	S Andrea	White	2021	Rovasenda (VC)
10	Organic	Baldo	White	2021	Rovasenda (VC)
11	Organic	Carnaroli	Brown	2022	Rovasenda (VC)
12	Organic	Carnaroli	Brown	2019	Olevano (PV)
13	Organic	Carnaroli	White	2020	Olevano (PV)
14	Organic	Rosa Marchetti	Brown	2022	Rovasenda (VC)
15	Organic	Carnaroli	White	2020	Olevano (PV)
16	Organic	Carnaroli	White	2022	Robbio (PV)
17	Organic	Rosa Marchetti	Brown	2022	Nicorvo (PV)
18	Organic	Rosa Marchetti	White	2022	Nicorvo (PV)
19	Conventional	Generic	Brown	2023	Commerce
20	Conventional	Arborio	White	2023	Commerce
21	Conventional	Arborio	White	2023	Commerce
22	Conventional	Carnaroli	White	2023	Commerce
23	Conventional	Arborio	White	2023	Commerce
24	Conventional	Carnaroli	White	2023	Commerce
25	Conventional	Arborio	White	2023	Commerce
26	Conventional	Carnaroli	White	2023	Commerce
27	Conventional	Carnaroli	White	2023	Commerce
28	Conventional	Arborio	White	2023	Commerce
29	Conventional	Arborio	White	2023	Commerce

30	Conventional	Carnaroli	White	2023	Commerce
31	Conventional	Carnaroli	White	2023	Commerce
32	Conventional	Arborio	White	2023	Commerce
33	Conventional	Generic	Brown	2023	Commerce
34	Organic	Generic	Brown	2023	Commerce
35	Conventional	Generic	Brown	2023	Commerce
36	Conventional	Generic	Brown	2023	Commerce
37	Conventional	Generic	Brown	2023	Commerce
38	Organic	Baldo	Brown	2023	Commerce

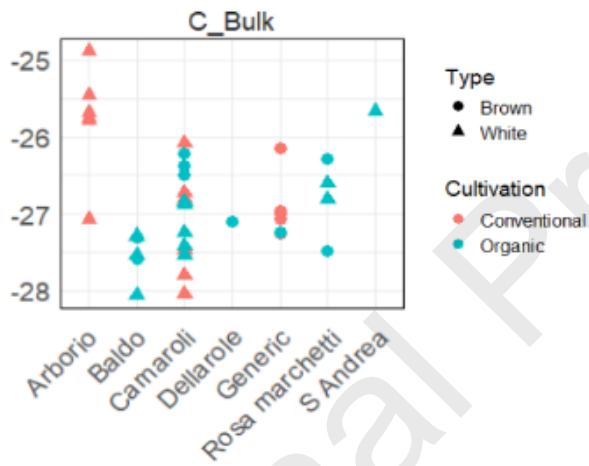


Figure A1. $\delta^{13}C_{bulk}$ variability across Italian rice varieties

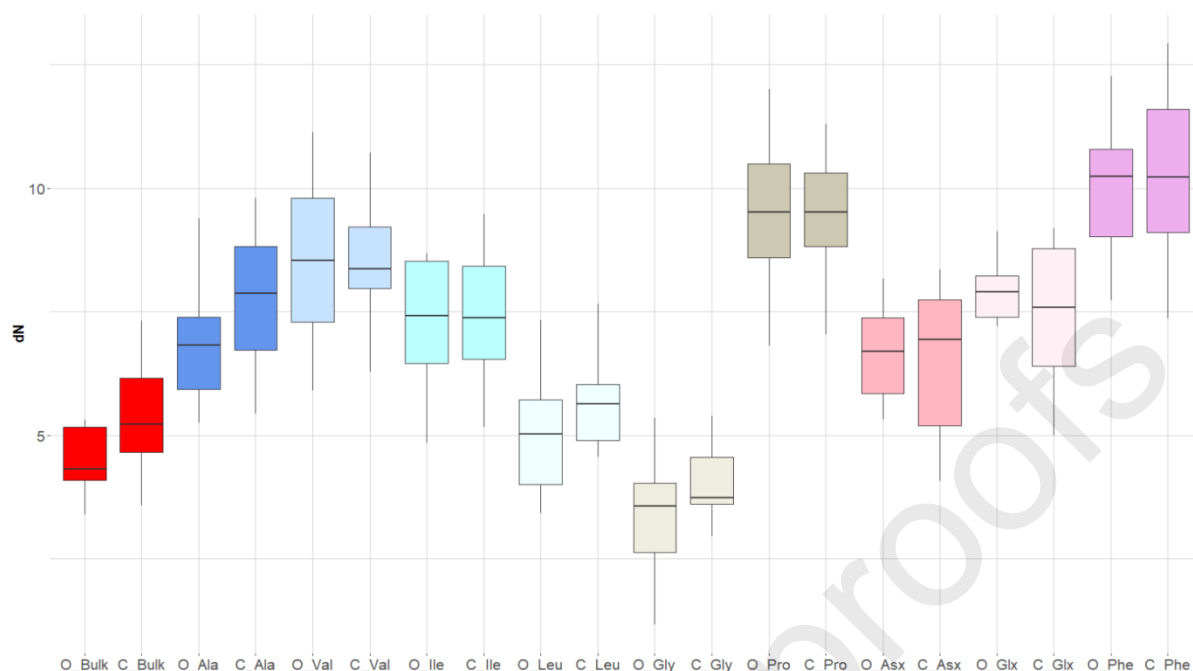


Figure A2. White rice $\delta^{15}\text{N}_{\text{AA}}$ values of organic (O) and conventional (C) cultivation.

Table A2. LDA confusion matrix and results

Confusion Matrix	Arborio_Conv	Baldo_Org	Carnaroli_Conv	Carnaroli_Org	Generic_OrgConv
Arborio_Conv	1	0	0	0	0
Baldo_Org	0	0	0	0	0
Carnaroli_Conv	0	0	1	0	0
Carnaroli_Org	0	1	0	1	0
Generic_OrgConv	0	0	0	0	1
Overall Statistics:					
Accuracy : 0.8					
95% CI : (0.2836, 0.9949)					
No Information Rate : 0.2					
P-Value [Acc > NIR] : 0.00672					
Kappa : 0.75					
Statistics by Class:					
	Class: Arborio_Conv	Class: Baldo_Org	Class: Carnaroli_Conv	Class: Carnaroli_Org	Class: Generic_OrgConv
Sensitivity	1	0	1	1	1

Specificity	1	1	1	0.75	1
Balanced Accuracy	1	0.5	1	0.875	1
Precision	1	0	1	0.5	1
Recall	1	0	1	1	1

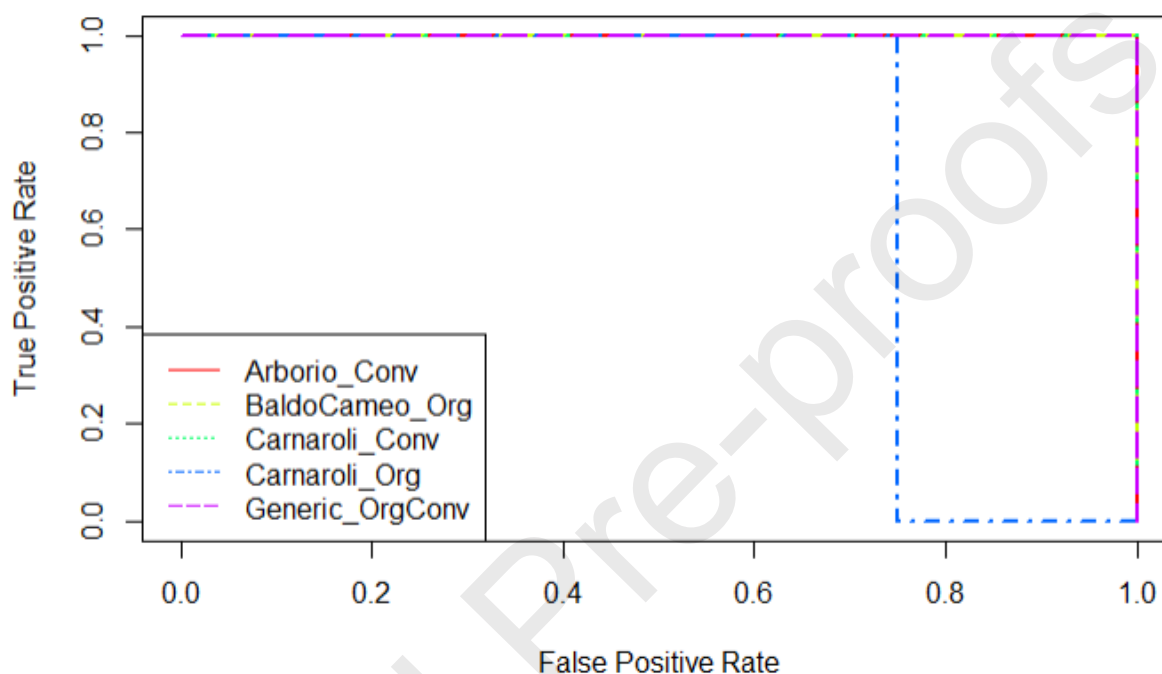


Figure A3. ROC curves obtained for all LDA classes.

Table A3. Terminology of LDA model interpretation

Kappa Coefficient	A measure of inter-rater agreement that assesses how well the predicted classifications agree with actual classifications, adjusting for chance. It ranges from -1 to 1, where higher values indicate better agreement.
No Information Rate (NIR)	The expected accuracy of a model if it always predicts the most common class. A model should perform significantly better than the NIR to demonstrate its effectiveness.
Area Under the Curve (AUC)	A metric derived from the Receiver Operating Characteristic (ROC) curve that represents the ability of the model to distinguish between classes. The AUC ranges from 0 to 1, with 1 indicating perfect classification ability.
Precision	The ratio of true positive predictions to the total predicted positives. It measures the accuracy of the positive predictions made by the model. High precision indicates fewer false positives.
Recall (Sensitivity)	The ratio of true positive predictions to the total actual positives. It reflects the model's ability to identify all relevant instances. High recall indicates fewer false negatives.

Specificity	The ratio of true negative predictions to the total actual negatives. It measures the model's ability to correctly identify negative instances. High specificity indicates fewer false positives.
Receiver Operating Characteristic (ROC) Curve	A graphical representation of a classifier's performance across different threshold values, plotting the true positive rate (sensitivity) against the false positive rate.

Table A4. Decision Tree confusion matrix and results

Confusion Matrix	Arborio_Conv	Baldo_Org	Carnaroli_Conv	Carnaroli_Org	Generic_OrgConv
Arborio_Conv	1	0	0	0	0
Baldo_Org	0	0	0	0	0
Carnaroli_Conv	0	1	1	0	0
Carnaroli_Org	0	0	0	1	0
Generic_OrgConv	0	0	0	0	1
Overall Statistics:					
Accuracy : 0.8					
95% CI : (0.2836, 0.9949)					
No Information Rate : 0.2					
P-Value [Acc > NIR] : 0.00672					
Kappa : 0.75					
Statistics by Class:					
	Class: Arborio_Conv	Class: Baldo_Org	Class: Carnaroli_Conv	Class: Carnaroli_Org	Class: Generic_OrgConv
Sensitivity	1	0	1	1	1
Specificity	1	1	0.75	1	1
Balanced Accuracy	1	0.5	0.875	1	1
Precision	1	0	0.5	1	1
Recall	1	0	1	1	1

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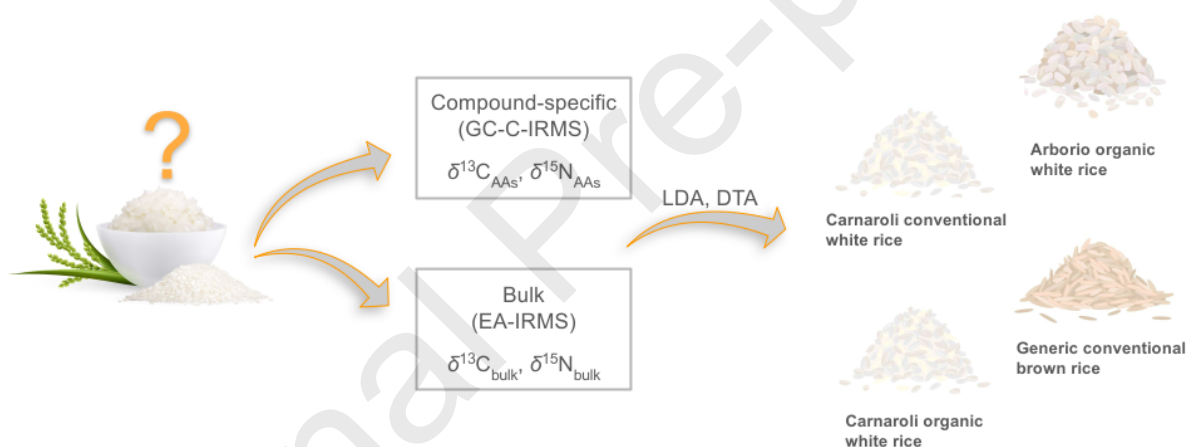
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Graphical Abstract



Highlights

- Simultaneous classification of variety and cultivation was achieved by CS-IRMS data
- Generic rice was separated from the premium Italian varieties with 100% accuracy
- $\delta^{15}\text{N}_{\text{AAs}}$ fluctuated significantly more than $\delta^{13}\text{C}_{\text{AAs}}$ across all variables

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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