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Increasing Amino Acids Content of White Wines with Enzymes Treatments

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Abstract: Wine's chemical structure is affected by many biochemical transformations during the winemaking process, which are catalysed by specific enzymes. These compounds participate in the formation of amino acids, which also have fundamental functions in the sensory quality of wine. Therefore, this research focuses on monitoring the effect of enzymes on amino acid concentration during the fermentation of Fetească regală and Sauvignon blanc wines. A total of 22 amino acids were quantified using an ultra-high liquid chromatography system coupled with mass spectrometry detection. Data indicated a major impact of the analysed variables (enzyme type and grape variety) on wine's characteristics. Considerable amounts of some essential amino acids, such as histidine, isoleucine, phenylalanine, and tryptophan, were found in samples treated with pectinases preparations. The administration of pectinases was more effective in the Fetească regală wines in the applied work conditions, although the β-glycosides generated the highest values for most amino acids in the Sauvignon blanc. Pectinases can provide more acceptable sensory characteristics of wine compared to β-glycosides in the applied work conditions (when they are applied in the pre-fermentation stage), while these samples generally showed the lowest intensity for some negative descriptors, such as phenolic, mineral or a bitter taste.

Keywords: beverages composition; fermentation; pectinases; β-glycosides; winemaking optimization

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1. Introduction

Enzymes are essential in food and beverages processes. Since the endogenous enzymes of grapes, yeasts and other microorganisms are present in must and wine in low quantities and their activity is not sufficient, commercial enzymes are often administrated as supplements [1]. Enzymes are involved in carbohydrate transformation reactions according to biocatalysts' general mechanism of action [2]. The enzymes and other nitrogen compounds from the grapes represent nutritional support for yeasts which, in turn, can be source of amino acids. The profile and concentration of these compounds in wines may be influenced by several factors, such as grape variety, cultivation technology (e.g., the application of nitrogen treatments) and winemaking protocols (e.g., fermentation process, the type of yeast), amination and transamination reactions of aldehydes and ketones, etc. [3].

Most amino acids are consumed from the must until the first 30 g of sugars have been fermented. In grape juice, significant quantities of proline, alanine and arginine are found, but of these, yeasts prefer alanine as a nutrient. During fermentation, the yeasts assimilate between 1 and 2 g/L of amino acids. Towards the end of the fermentation, the yeasts release significant, but variable, amounts of different amino acids, predominantly proline (about half of their total) [4].

Some studies [5–7] confirmed the positive impact of enzyme administration in beverage industries by obtaining a higher control over the quality of operations, optimising pressing, centrifugation and clarification processes, reducing consumption energy, increasing wine stability and contributing to the extraction of phenolic or volatile compounds in wines. Pectinases, glucanase and glycosidase are the most widely used in winemaking. Enzyme preparations are generally mixtures of products with diverse enzymatic activities. It is known that enzymes can be a source of nutrients for yeast and can influence their activity [2]. Yeast can also release certain enzymes under certain technological conditions. Their efficiency depends on the temperature level and pH value. Thus, the optimum temperature for administrating pectinases ranges from 10 °C to 55 °C. β-Glycosidase can be used above 15 °C and require a longer incubation time. Enzyme activity in wine is not usually inhibited by the use of sulphur dioxide, while the enzyme treatment should be administrated in higher doses if pH values are low. In red wines, the inhibition of enzymatic activity may occur under the action of phenolic compounds, which may determine an increase in the dose of administered product. An alcohol concentration of higher than 14% vol. does not affect the enzyme's activity [2].

The sensory profile is essential in defining wine's quality and has a decisive impact on consumer acceptance. Amino acid compounds serve as nutritional support for yeasts during fermentation and are essential when defining the sensory complexity of wines. Amino acid concentrations are dependent on grape variety, cultivation and winemaking practices, climatic conditions, etc. [2]. They are the main nitrogen compounds in musts and wines, representing about 20–30% of their total in white wine and up to 50% in red wine. Important proportions of amino acids can result from yeast metabolism [8]. Amino acids manifest major functions in the wine's volatile compounds synthesis (these are metabolic precursors of higher alcohols, volatile acids and esters). Moreover, amino acids represent key elements in differentiating grapes and wines according to variety or cultivation area [8]. Soufleros et al. [9] confirmed that wine's amino acid profile, correlated to the statistical analysis, can offer sufficient information to classify wine according to the mentioned variables. Insufficient amounts of these compounds can lead to incomplete fermentation and unwanted modifications in wine structure, such as hydrogen sulphide production or increased acetic acid content [4]. Additionally, the concentration of amino acids represents a significant criterion for classifying wines [8,10].

The most common amino acids found in wine are as follows: proline, arginine, alanine, glycine, aspartic acid, glutamic acid, glutamine, serine, threonine, methionine, phenylalanine, 4-hydroxyproline [2]. The influence of different oenological treatments on the amino acid level in wine is of interest for researchers. The effect of some enzymatic preparations on the evolution of different wine compounds (including amino acids) requires more research. Given the importance of *terroir* on the amino acid content, it should be noted that no paper was found the evolution of these compounds in Fetească regală and Sauvignon blanc varieties from Romanian vineyards (NE region). This study is an extension of previous research attempting to clarify the impact of enzymes on the quality of white wines [11–13].

Therefore, this research focuses on monitoring the concentration of amino acids during the fermentation of white wines treated with enzymes (pectinases and β-glycosides). This work refers to the influence of enzymatic preparations administered before the alcoholic fermentation stage, even if the producer's recommendation is to use them at other stages of the winemaking process. The enzymes were randomly selected due to the numerous assortments in which they are found.

2. Materials and Methods

2.1. Chemicals

Standard solutions and reagents were of HPLC-grade purity, and all chemicals were of analytical grade, purchased from Merck KgaA (Darmstadt, Germany): acetonitrile, propan-2-ol; ammonium formate, formic acid, hydrochloric acid. 22 amino acids standard were used, as followed: L-aspartic acid, L-glutamic acid, L-leucine, L-isoleucine, Trans-4-L-

hydroxyproline, L-methionine, L-tyrosine, L-threonine, L-valine, L-tryptophan, L-alanine, L-proline, L-asparagine, L-glycine, L-serine, L-glutamine, L-cysteine, L-cystine, L-lysine, L-arginine, L-histidine, L-phenylalanine.

2.2. Grapes and Winemaking Procedures

A Romanian autochthonous and an international grape variety (Fetească regală and Sauvignon blanc), which are extremely widespread in Romanian vineyards, were selected for this purpose. The grapes were harvested in October 2018 (with 210 g/L sugars in Fetească regală grapes and 250 g/L in Sauvignon blanc) from Copou-Iași vineyard (47°10′ north latitude 27°35′ east longitude), they were destemmed and pressed, and the extracted must was divided into six aliquots in 50-L glass vessels. *Saccharomyces* yeast (*Levulia® esperide*, AEB, San Polo, Italy) was inoculated at a dose of 20 g/hL and 30 g/hL yeast nutrient (FERMOPLUS® CH, AEB, San Polo, Italy) was applied to each container, both dissolved in must. Different commercial enzymes (based on pectinase and β-glycosidase activities) were administrated before alcoholic fermentation, as follows: Endozym Thiol®, AEB, San Polo, Italy—**V1**; Endozym® β-Split®, AEB, San Polo, Italy—**V2**; Zymovarietal® aroma G, SODINAL, Plovdiv, Bulgaria—**V3**; Endozym® Ice, AEB, San Polo, Italy—**V4**; Zimarom®, BSG WINE, Napa, California—**V5** and no enzyme—**V6**), at a dose of 3 g/hL for powder products (Endozym® β-Split, Zymovarietal® aroma G, Zimarom®) and 3 mL/hL for the liquid ones (Endozym Thiol®, Endozym® Ice). The dosages were in line with the producer's instructions and current OIV regulations [14]. After enzymatic treatment, fermentation was carried out at 16–18 °C for about 20 days and samples were constantly collected every three days and kept at −20 °C until analysis. When the alcoholic fermentation ended, all variants were sulphated (1.5 mL/L SO_2 6%), filtered (through 0.45-µm sterile membrane filters), bottled and stored under controlled conditions (constant temperature at 8 °C, dark, stable humidity 70–80%), and analysed after approximately 6 months.

The resulted wines were dry, with over 12.7% vol. in Fetească regală samples (and 1.3–1.8 g/L residual sugar) and exceeded 16.2% vol. in those obtained from the Sauvignon blanc variety (and 1.9–2.7 g/L residual sugar) (Figure 1).

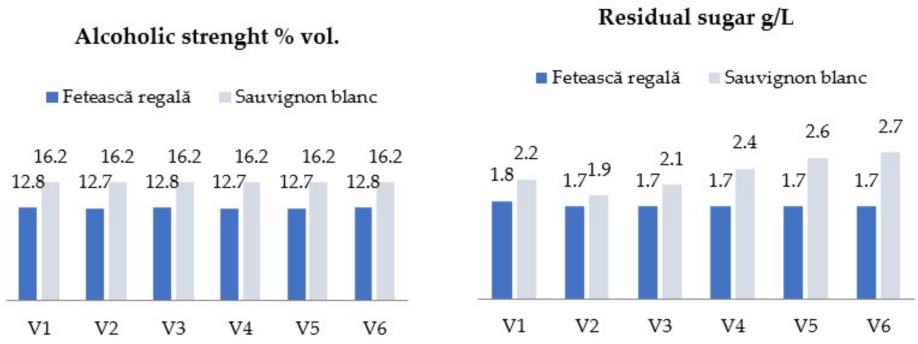

Figure 1. Alcoholic strength and residual sugar of experimental wines. V1—Endozym Thiol®, AEB; V2—Endozym β-Split®, AEB; V3—Zymovarietal aroma G®, SODINAL; V4—Endozym Ice®, AEB; V5—Zimarom®, BSG WINE; V6—control sample, no enzymes.

2.3. Amino Acid Identification and Quantification

Amino acid identification and quantification was performed using UHPLC with mass spectrometry detection. For this, an UltiMate™ 3000 UHPLC system (Thermo Scientific™, Waltham, MA, USA) was used, with the following components: pressure pump, UltiMate™ LPG-3400RS model (Thermo Scientific™, Waltham, MA, USA); autoinjector with interchangeable valves interface module; thermostatic column compartment, UltiMate™ TCC-3000RS (Thermo Scientific™, Waltham, MA, USA); Intrada Amino Acid chromatographic column (Imtakt Corp., Kyoto, Japan), measuring 150 × 3 mm, 3 µm particle size, 130 Å); detection system, represented by TSQ Quantum Access Max mass spectrophotometer with triple quadrupole (Thermo Scientific™, Waltham, MA, USA);

Xcalibur™ software (Thermo Scientific™, Waltham, MA, USA), for efficient data processing and the delivery of results. The mobile phase consisted of solutions A and B, obtained as follows: (1) eluent A, obtained by mixing propan-2-ol (74% *v/v*), acetonitrile (10% *v/v*), ammonium formate, 25 mM solution (15.8% *v/v*) and formic acid (0.2% *v/v*); (2) eluent B, obtained by 100 mM ammonium formate (80% *v/v*) and 20 acetonitrile (% *v/v*). For the calibration and quantification of these compounds, a series of standard solutions were prepared to verify the linearity range of the method. For every amino acid, the linearity range concentrations between 100 mg/L and 1 mg/L were prepared according to Table S1 in the Supplementary Materials. The pump ensures a constant flow of approximately 0.35 mL per min. Every amino acid in the series had the linearity range of minimum 0.998 correlation coefficient, except for cysteine, cystine, and lysine, whose R^2 was a minimum of 0.99 in relation to the concentrations included in the study.

A total of 5 µL of prepared solution was injected in the chromatographic column; the oven temperature was set at 45 °C for separation. The following working conditions were applied: potential discharge ionization source type H-ESI II (heated electron spray ionization): 3 kV; ionization temperature: 350 °C; nebulizer gas pressure (N2): 35 psi; auxiliary gas pressure (N2): 10 psi; the pre-set temperature of the capillary column: 380 °C; the capillary compensation voltage: 35 V; positive polarization source (+).

For analysis, wine aliquots were centrifuged (10.000 rpm, 5 min) and filtered before injection.

2.4. Sensory Evaluation

The sensory analysis of the experimental samples obtained was performed in accordance with the specifications indicated by the standard ISO 8589: 2010 [13], ISO 3591: 1997 [14] and the OIV recommendations [15] regarding the tasting conditions. The preparation of the samples to be analyzed consisted of bringing them to the same temperature (10–12 °C). The tasting session was organized in the first part of the day to ensure a better perception of the studied descriptors. The sensory profile of the wines was evaluated by a panel of 20 licensed tasters, consisting of 12 men and 8 women. The evaluation of the sensory characteristics was performed following key descriptors specific to white grape varieties and by giving marks from 0 to 5, depending on the intensity of the analyzed sensory qualities [15].

2.5. Statistical Analysis

Anova one-way, Pearson correlation and post-hoc Fisher LSD analysis was performed using STATGRAPHICS 19® software. To better highlight the results of the sensory analysis, a map of the intensity of the sensory descriptors was created using the online Heatmapper server (developed in the Wishart Research Group at the University of Alberta, Edmonton, AB, Canada). All determinations were run in triplicate and values were averaged.

3. Results

3.1. The Influence of Enzymes on Amino Acid Concentration

Regarding the HPLC analysis, 22 amino acids were identified. Figures 2 and 3 present the content of analyzed compounds during different fermentation stages. Tables 1 and 2 show amino acid levels in resulted wines, while Tables 3 and 4 present the correlation between the analysed compounds. Amino acids concentrations were differentiated according to fermentation stage or applied enzymes.

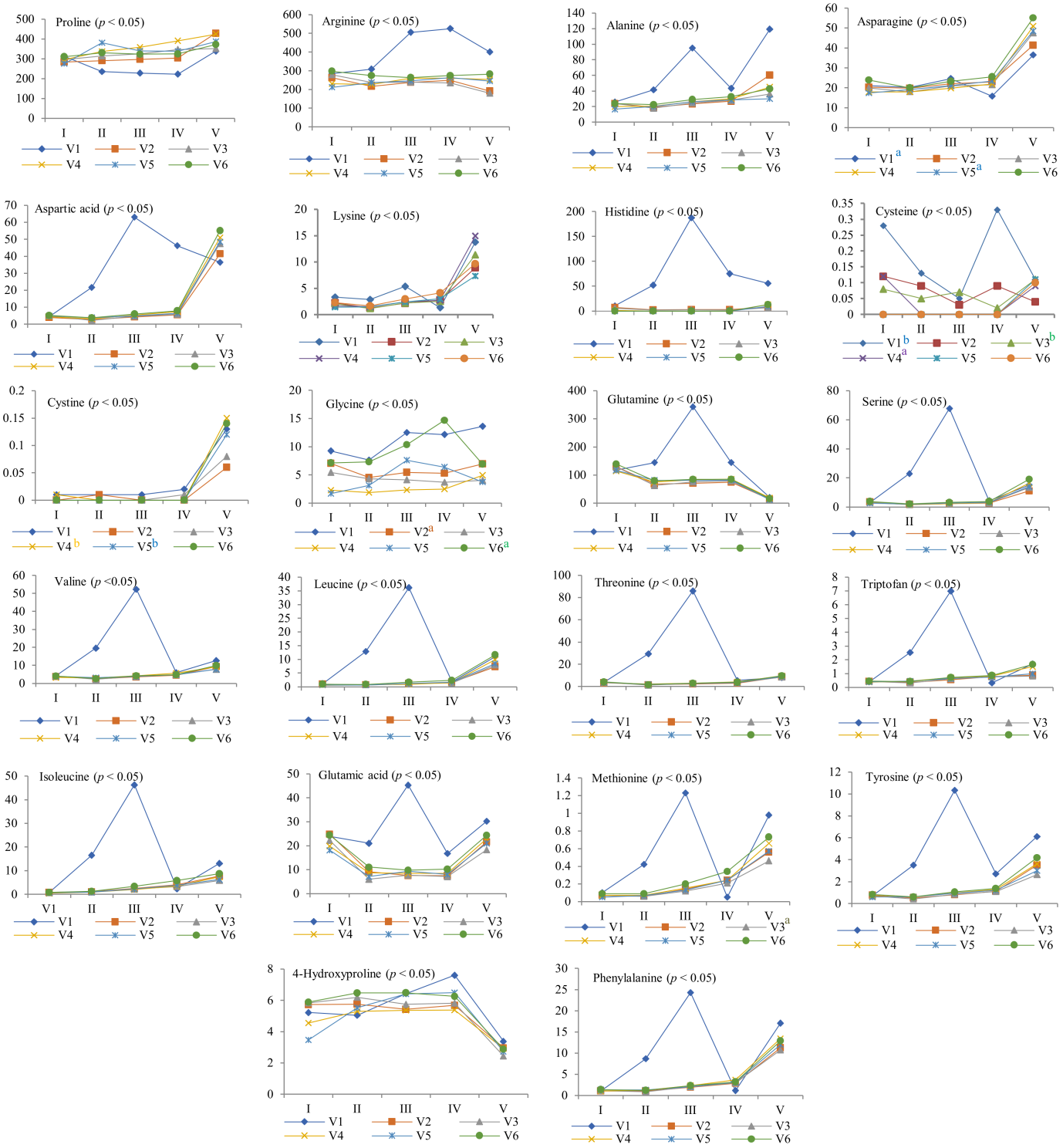

Figure 2. The influence of enzymatic treatments on the evolution of amino acids during the fermentation of Fetească regală samples (mg/L). I, II, III, IV—fermentation stage (ziua 1, 3, 6, 9); V—Final sample (stabilized wine); V1—Endozym Thiol®, AEB; V2—Endozym β-Split®, AEB; V3—Zymovarietal aroma G®, SODINAL; V4—Endozym Ice®, AEB; V5—Zimarom®, BSG WINE; V6—control sample, no enzymes. The superscript letters indicate homogeneous groups, between which there is no statistically significant difference ($p > 0.05$), in correlation with the Fisher LSD test.

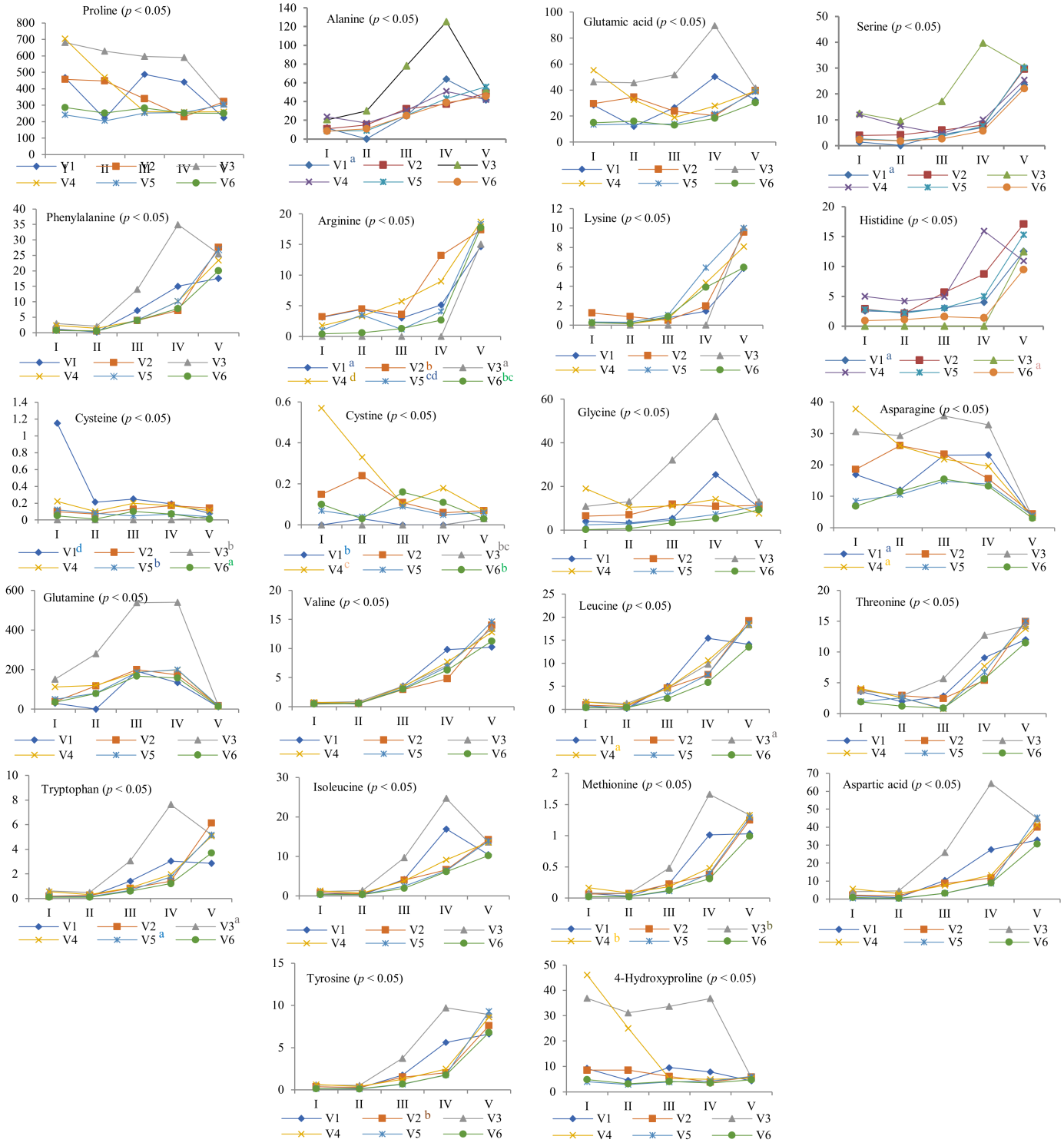

Figure 3. The influence of enzymatic treatments on the evolution of amino acids during the fermentation of Sauvignon blanc samples (mg/L). I, II, III, IV—fermentation stage (ziua 1, 3, 6, 9); V—Final sample (stabilized wine); V1—Endozym Thiol®, AEB; V2—Endozym β-Split®, AEB; V3—Zymovarietal aroma G®, SODINAL; V4—Endozym Ice®, AEB; V5—Zimarom®, BSG WINE; V6—control sample, no enzymes. The superscript letters indicate homogeneous groups, between which there is no statistically significant difference ($p > 0.05$) in correlation with the Fisher LSD test.

Table 1. Evaluation of some amino acid concentrations in the resulting Fetească regală wines (mg/L).

C	V1	V2	V3	V4	V5	V6	*p*	C	V1	V2	V3	V4	V5	V6	*p*
Arg	400.27 ± 0.28 *	191.83 ± 1.08 *	180.50 ± 0.22 *	252.95 ± 0.83 *	245.29 ± 0.28 *	282.29 ± 1.29 *	0.0000	Leu	10.98 ± 0.00 *	7.27 ± 0.01 *	7.91 ± 0.00 *	9.89 ± 0.01 *	8.40 ± 0.00 *	11.66 ± 0.01 *	0.0000
Lys	13.76 ± 0.00 *	8.88 ± 0.05 *	11.36 ± 0.01 *	14.93 ± 0.06 *	7.33 ± 0.01 *	9.65 ± 0.03 *	0.0000	Thr	8.20 ± 0.00 *	8.71 ± 0.01 *	8.53 ± 0.00 *	9.72 ± 0.01 *	9.23 ± 0.00 *	9.68 ± 0.01 *	0.0000
His	55.71 ± 0.19 *	7.79 ± 0.04 *	6.85 ± 0.02 *	12.89 ± 0.05 *	9.38 ± 0.00 *	13.14 ± 0.08 *	0.0000	Trp	1.70 ± 0.00 *	0.88 ± 0.00 *	0.82 ± 0.00 *	1.51 ± 0.00 *	0.99 ± 0.00 *	1.66 ± 0.00 *	0.0000
Cys	0.11 ± 0.00[b]	0.04 ± 0.00 *	0.11 ± 0.00 [b]	0.09 ± 0.00 [a]	0.11 ± 0.00 [b]	0.10 ± 0.00 *	0.0000	Ile	13.09 ± 0.00 *	7.70 ± 0.03 *	5.89 ± 0.00 *	7.41 ± 0.01 *	6.28 ± 0.00 *	8.72 ± 0.01 *	0.0000
Cystine	0.13 ± 0.00 *	0.06 ± 0.00 *	0.08 ± 0.00 *	0.15 ± 0.00 [b]	0.12 ± 0.00 [a]	0.14 ± 0.00 *	0.0000	Glu	30.27 ± 0.02 *	21.68 ± 0.05 *	18.35 ± 0.01 *	22.99 ± 0.03 *	21.03 ± 0.01 *	24.37 ± 0.01 *	0.0000
Gly	13.60 ± 0.01 *	6.95 ± 0.02 [a]	4.06 ± 0.02 *	4.93 ± 0.01 *	3.74 ± 0.00 *	6.91 ± 0.02 [a]	0.0000	Met	0.98 ± 0.00 *	0.56 ± 0.00 *	0.46 ± 0.00[a]	0.66 ± 0.00 *	0.57 ± 0.00 *	0.73 ± 0.00 *	0.0000
Asn	36.41 ± 0.00 *	41.37 ± 0.02 *	47.48 ± 0.01 *	50.86 ± 0.04 *	48.43 ± 0.00 *	55.06 ± 0.01 *	0.0000	Asp	18.84 ± 0.03 [a]	22.84 ± 0.07 *	18.15 ± 0.01 *	22.73 ± 0.03 *	18.88 ± 0.02 [a]	29.43 ± 0.05 *	0.0000
Ala	119.30 ± 0.00 *	60.35 ± 0.13 *	36.17 ± 0.04 *	44.96 ± 0.03 *	29.89 ± 0.02 *	42.84 ± 0.01 *	0.0000	Tyr	6.13 ± 0.01 *	3.50 ± 0.01 *	2.65 ± 0.00 *	3.63 ± 0.01 *	3.01 ± 0.00 *	4.18 ± 0.00 *	0.0000
Gln	20.78 ± 0.02 *	14.05 ± 0.03 *	13.29 ± 0.00 *	18.43 ± 0.02 *	12.43 ± 0.00 *	15.75 ± 0.02 *	0.0000	Phe	17.05 ± 0.00 *	11.27 ± 0.03 *	10.75 ± 0.00 *	13.41 ± 0.02 *	12.00 ± 0.00 *	12.89 ± 0.01 *	0.0000
Ser	15.04 ± 0.00 *	10.99 ± 0.01 *	13.32 ± 0.01 *	16.39 ± 0.00 *	13.94 ± 0.01 *	19.08 ± 0.02 *	0.0000	Pro	338.06 ± 0.03 *	429.08 ± 1.17 *	351.73 ± 0.04 *	424.47 ± 0.65 *	387.17 ± 0.06 *	372.46 ± 0.56 *	0.0000
Val	12.70 ± 0.00 *	9.35 ± 0.02 *	7.86 ± 0.01 *	9.17 ± 0.02 *	7.82 ± 0.00 *	9.97 ± 0.01 *	0.0000	Hyp	3.38 ± 0.00 *	2.97 ± 0.01 *	2.44± 0.00 *	2.92± 0.01 *	2.73± 0.00 *	2.88± 0.01 *	0.0000

Arg—Arginine; Lys—Lysine; His—Histidine; Cys—Cysteine; Cystine; Gly—Glycine; Asn—Asparagine; Ala—Alanine; Gln—Glutamine; Ser—Serine; Val—Valine; Leu—Leucine; Thr—Threonine; Trp—Tryptophan; Ile—Isoleucine; Glu—Glutamic acid; Met—Methionine; Asp—Aspartic acid; Tyr—Tyrosine; Phe—Phenylalanine; Pro—Proline; Hyp—4-Hydroxyproline. C-identified compounds; V1-Endozym Thiol®, AEB; V2-Endozym β-Split®, AEB; V3-Zymovarietal aroma G®, SODINAL; V4-Endozym Ice®, AEB; V5-Zimarom®, BSG WINE; V6-control sample, no enzymes. The results constitute the average of obtained values plus standard deviation. All samples were analysed in triplicate. The superscript letters indicate diverse homogeneous groups, between which there is no statistically significant difference ($p > 0.05$) in correlation with the Fisher LSD test; *—significant difference compared to all the analysed variants; ns—insignificant ($p > 0.05$).

Table 2. Evaluation of some amino acid concentrations in resultant Sauvignon blanc wines (mg/L).

C	V1	V2	V3	V4	V5	V6	*p*	C	V1	V2	V3	V4	V5	V6	*p*
Arg	14.67 ± 0.01 ^{a}	17.39 ± 0.01 ^{b}	15.07 ± 0.03 ^{a}	18.75 ± 0.03 ^{d}	18.32 ± 0.01 ^{cd}	17.76 ± 0.05 ^{bc}	0.0000	Leu	14.06 ± 0.00 *	19.23 ± 0.03 *	18.30 ± 0.01 ^{a}	18.33 ± 0.00 ^{a}	18.61 ± 0.05 *	13.51 ± 0.01 *	0.0000
Lys	5.83 ± 0.00 *	9.56 ± 0.00 *	10.03 ± 0.00 *	8.08 ± 0.03 *	9.99 ± 0.01 *	5.93 ± 0.00 *	0.0000	Thr	11.99 ± 0.00 *	14.93 ± 0.01 *	14.22 ± 0.01 *	13.74 ± 0.00 *	15.00 ± 0.04 *	11.47 ± 0.01 *	0.0000
His	12.53 ± 0.01 ^{a}	17.09 ± 0.01 *	12.49 ± 0.04 ^{a}	10.93 ± 0.04 *	15.30 ± 0.03 *	9.46 ± 0.00 *	0.0000	Trp	2.86 ± 0.00 *	6.12 ± 0.00 *	5.17 ± 0.00 ^{a}	5.06 ± 0.00 *	5.17 ± 0.02 ^{a}	3.70 ± 0.00 *	0.0000
Cys	0.08 ± 0.00 ^{d}	0.14 ± 0.00 *	0.04 ± 0.00 ^{b}	0.11 ± 0.00 *	0.04 ± 0.00 ^{b}	0.01 ± 0.00 ^{a}	0.0000	Ile	10.45 ± 0.00 *	14.22 ± 0.02 *	13.64 ± 0.01 *	13.56 ± 0.00 *	13.86 ± 0.04 *	10.18 ± 0.01 *	0.0000
Cystine	0.03 ± 0.00 ^{b}	0.07 ± 0.00 *	0.03 ± 0.00 ^{bc}	0.07 ± 0.00 ^{c}	0.06 ± 0.00 *	0.03 ± 0.00 ^{b}	0.0000	Glu	32.12 ± 0.01 *	39.63 ± 0.04 *	41.26 ± 0.01 *	39.18 ± 0.01 *	38.63 ± 0.09 *	30.29 ± 0.01 *	0.0000
Gly	10.51 ± 0.00 *	11.29 ± 0.01 *	12.88 ± 0.01 *	7.60 ± 0.01 *	11.10 ± 0.02 *	9.25 ± 0.00 *	0.0000	Met	1.03 ± 0.00 *	1.25 ± 0.00 *	1.33 ± 0.00 ^{b}	1.33 ± 0.00 ^{b}	1.29 ± 0.00 *	0.99 ± 0.00 *	0.0000
Asn	3.11 ± 0.00 ^{a}	4.36 ± 0.00 *	3.35 ± 0.00 *	3.17 ± 0.00 ^{a}	3.85 ± 0.01 *	2.97 ± 0.00 *	0.0000	Asp	32.83 ± 0.03 *	40.05 ± 0.05 *	44.76 ± 0.01 *	41.40 ± 0.04 *	45.38 ± 0.10 *	30.63 ± 0.02 *	0.0000
Ala	41.65 ± 0.00 ^{a}	48.77 ± 0.03 *	53.04 ± 0.04 *	41.84 ± 0.00 *	55.74 ± 0.06 *	45.55 ± 0.02 *	0.0000	Tyr	6.61 ± 0.01 *	7.60 ± 0.01 ^{b}	8.93 ± 0.00 *	8.60 ± 0.01 *	9.30 ± 0.02 *	6.78 ± 0.00 *	0.0000
Gln	11.07 ± 0.00 *	17.27 ± 0.00 *	16.10 ± 0.00 *	13.33 ± 0.00 *	17.77 ± 0.04 *	13.21 ± 0.01 *	0.0000	Phe	17.61 ± 0.01 *	27.67 ± 0.03 *	25.53 ± 0.00 *	23.46 ± 0.02 *	26.89 ± 0.07 *	20.06 ± 0.02 *	0.0000
Ser	23.60 ± 0.02 ^{a}	29.58 ± 0.00 *	30.37 ± 0.01 *	25.44 ± 0.01 *	30.07 ± 0.06 *	22.04 ± 0.01 *	0.0000	Pro	224.07 ± 0.33 *	321.85 ± 0.13 *	307.06 ± 0.04 *	260.30 ± 0.37 *	303.37 ± 0.35 *	250.75 ± 0.11 *	0.0000
Val	10.25 ± 0.00 *	13.98 ± 0.01 *	13.44 ± 0.00 *	12.79 ± 0.00 *	14.62 ± 0.03 *	11.27 ±0.02 *	0.0000	Hyp	4.33 ± 0.01 *	5.86 ± 0.00 *	5.64 ± 0.00 *	5.36 ± 0.01 *	6.11 ± 0.01 *	4.74 ± 0.00 *	0.0000

Arg—Arginine; Lys—Lysine; His—Histidine; Cys—Cysteine; Cystine; Gly—Glycine; Asn—Asparagine; Ala—Alanine; Gln—Glutamine; Ser—Serine; Val—Valine; Leu—Leucine; Thr—Threonine; Trp—Tryptophan; Ile—Isoleucine; Glu—Glutamic acid; Met—Methionine; Asp—Aspartic acid; Tyr—Tyrosine; Phe—Phenylalanine; Pro—Proline; Hyp—4-Hydroxyproline. C-identified compounds; V1-Endozym Thiol®, AEB; V2-Endozym β-Split®, AEB; V3-Zymovarietal aroma G®, SODINAL; V4-Endozym Ice®, AEB; V5-Zimarom®, BSG WINE; V6-control sample, no enzymes. The results constitute the average of obtained values plus standard deviation. All samples were analysed in triplicate. The superscript letters indicate homogeneous groups, between which there is no statistically significant difference ($p > 0.05$) in correlation with the Fisher LSD test; *—significant difference compared to all the analysed variants; ns—insignificant ($p > 0.05$).

Table 3. Pearson correlation of amino acid content in resultant Fetească regală wines.

	Arg	Lys	His	Cys	Cystine	Gly	Asn	Ala	Gln	Ser	Val	Leu	Thr	Trp	Ile	Glu	Met	Asp	Tyr	Phe	Pro	Hyp
Arg	1	0.4299	0.9279	0.4226	0.6283	0.8449	−0.3806	0.7935	0.8163	0.4627	0.8817	0.7812	−0.1941	0.8298	0.9175	0.9603	0.9781	0.0061	0.9410	0.9749	−0.5101	0.8262
Lys	0.4299	1	0.5063	0.2042	0.4422	0.3961	−0.1842	0.4746	0.8283	0.2915	0.4863	0.4204	−0.0752	0.5527	0.4433	0.4474	0.4722	−0.1219	0.4627	0.5869	−0.1298	0.3847
His	0.9279	0.5063	1	0.3218	0.3492	0.9330	−0.6637	0.9401	0.818	0.1646	0.9132	0.5591	−0.5218	0.6393	0.9488	0.9186	0.9197	−0.2447	0.9433	0.9499	−0.5722	0.8245
Cys	0.4226	0.2042	0.3218	1	0.8245	0.0313	0.2143	0.0014	0.1565	0.5059	0.0417	0.4637	0.0031	0.3167	0.1227	0.1635	0.2535	−0.2861	0.1517	0.335	−0.7583	−0.1527
Cystine	0.6283	0.4422	0.3492	0.8245	1	0.1704	0.3969	0.09481	0.5772	0.8588	0.3295	0.8312	0.5706	0.8332	0.3385	0.5132	0.5747	0.3099	0.4178	0.5969	−0.1351	0.3401
Gly	0.8449	0.3961	0.933	0.0313	0.1704	1	−0.6954	0.9749	0.7761	0.1037	0.9782	0.5237	−0.5371	0.6123	0.9847	0.9242	0.9016	−0.02481	0.9657	0.8643	−0.4464	0.8899
Asn	−0.3806	−0.1842	−0.6637	0.2143	0.3969	−0.6954	1	−0.809	−0.3608	0.6199	−0.561	0.2213	0.8767	0.0785	0.5907	−0.4665	−0.4075	0.5925	−0.5346	−0.4642	0.2273	−0.5855
Ala	0.7935	0.4746	0.9401	0.0014	0.0948	0.9749	-0.809	1	0.784	−0.0516	0.9354	0.3832	−0.6337	0.5083	0.9449	0.8733	0.8441	−0.207	0.9202	0.8514	−0.3997	0.8738
Gln	0.8163	0.8283	0.8180	0.1565	0.5772	0.7761	−0.3608	0.784	1	0.4113	0.8632	0.6953	−0.119	0.8364	0.8372	0.8658	0.8728	0.06532	0.8610	0.9091	−0.2157	0.8106
Ser	0.4627	0.2915	0.1646	0.5059	0.8588	0.1037	0.6199	−0.0516	0.4113	1	0.2695	0.9002	0.6276	0.8073	0.2522	0.3783	0.3783	0.6335	0.3182	0.3810	−0.2376	0.1455
Val	0.8817	0.4863	0.9132	0.0417	0.3295	0.9782	−0.561	0.9354	0.8632	0.2695	1	0.6561	−0.363	0.7524	0.9921	0.9655	0.9505	0.1189	0.9888	0.9062	−0.3732	0.9207
Leu	0.7812	0.4204	0.5591	0.4637	0.8312	0.5237	0.2213	0.3832	0.6953	0.9002	0.6561	1	0.3092	0.9628	0.6481	0.7367	0.7877	0.5096	0.6985	0.7192	−0.3945	0.5236
Thr	−0.1941	−0.07526	−0.5218	0.0031	0.5706	−0.5371	0.8767	−0.6337	−0.119	0.6276	−0.363	0.3092	1	0.2775	−0.4064	−0.2188	−0.1855	0.6693	−0.3311	−0.2367	0.5187	−0.2325
Trp	0.8298	0.5527	0.6393	0.3167	0.8332	0.6123	0.0785	0.5083	0.8364	0.8073	0.7524	0.9628	0.2775	1	0.7322	0.8292	0.8628	0.4595	0.7838	0.8172	−0.2438	0.6855
Ile	0.9175	0.4433	0.9488	0.1227	0.3385	0.9847	0.5907	0.9449	0.8372	0.2522	0.9921	0.6481	−0.4064	0.7322	1	0.9742	0.9629	0.0409	0.9960	0.9280	−0.4448	0.9126
Glu	0.9603	0.4474	0.9186	0.1635	0.5132	0.9242	−0.4665	0.8733	0.8658	0.3783	0.9655	0.7367	−0.2188	0.8292	0.9742	1	0.9949	0.1191	0.9896	0.9632	−0.3519	0.9379
Met	0.9781	0.4722	0.9197	0.2535	0.5747	0.9016	−0.4075	0.8441	0.8728	0.3783	0.9505	0.7877	−0.1855	0.8628	0.9629	0.9949	1	0.1228	0.9825	0.9731	−0.408	0.9003
Asp	0.0061	−0.1219	−0.2447	−0.2861	0.3099	−0.0248	0.5925	−0.207	0.0653	0.6335	0.1189	0.5096	0.6693	0.4595	0.0409	0.1191	0.1228	1	0.0772	−0.06445	0.3031	0.1057
Tyr	0.9410	0.4627	0.9433	0.1517	0.4178	0.9657	−0.5346	0.9202	0.8610	0.3182	0.9888	0.6985	−0.3311	0.7838	0.9960	0.9896	0.9825	0.0772	1	0.9492	−0.4217	0.9210
Phe	0.9749	0.5869	0.9499	0.335	0.5969	0.8643	−0.4642	0.8514	0.9091	0.381	0.9062	0.7192	−0.2367	0.8172	0.928	0.9632	0.9731	−0.0644	0.9492	1	−0.4191	0.8689
Pro	−0.5101	−0.1298	−0.5722	−0.7583	−0.1351	−0.4464	0.2273	−0.3997	−0.2157	−0.2376	−0.3732	−0.3945	0.5187	−0.2438	−0.4448	−0.3519	−0.408	0.3031	−0.4217	−0.4191	1	
Hyp	0.8262	0.3847	0.8245	−0.1527	0.3401	0.8899	−0.5855	0.8738	0.8106	0.1455	0.9207	0.5236	−0.2325	0.6855	0.9126	0.9379	0.9003	0.1057	0.9210	0.8689	−0.0619	1

Arg—Arginine; Lys—Lysine; His—Histidine; Cys—Cysteine; Cystine; Gly—Glycine; Asn—Asparagine; Ala—Alanine; Gln—Glutamine; Ser—Serine; Val—Valine; Leu—Leucine; Thr—Threonine; Trp—Tryptophan; Ile—Isoleucine; Glu—Glutamic acid; Met—Methionine; Asp—Aspartic acid; Tyr—Tyrosine; Phe—Phenylalanine; Pro—Proline; Hyp—4-Hydroxyproline. V1—Endozym Thiol®, AEB; V2—Endozym β-Split®, AEB; V3—Zymovarietal aroma G®, SODINAL; V4—Endozym Ice®, AEB; V5—Zimarom®, BSG WINE; V6—control sample, no enzymes.

Table 4. Pearson correlation of amino acid content in resultant Sauvignon blanc wines.

	Arg	**Lys**	**His**	**Cys**	**Cystine**	**Gly**	**Asn**	**Ala**	**Gln**	**Ser**	**Val**	**Leu**	**Thr**	**Trp**	**Ile**	**Glu**	**Met**	**Asp**	**Tyr**	**Phe**	**Pro**	**Hyp**
Arg	1	0.1757	0.01649	0.1112	0.7199	−0.6112	0.222	0.05874	0.3203	−0.00094	0.4465	0.3242	0.2758	0.4372	0.3284	0.1306	0.2678	0.1887	0.3201	0.3869	0.208	0.4394
Lys	0.1757	1	0.6611	0.1966	0.4966	0.5425	0.708	0.7977	0.9113	0.9763	0.9527	0.9316	0.9593	0.8843	0.9407	0.9275	0.8868	0.9423	0.85	0.953	0.9307	0.9548
His	0.0164	0.6611	1	0.5205	0.5363	0.5406	0.9549	0.5231	0.7175	0.7515	0.6542	0.6542	0.7771	0.6225	0.6481	0.5365	0.4191	0.5066	0.2882	0.6949	0.7082	0.658
Cys	0.1112	0.1966	0.5205	1	0.7	−0.1681	0.5277	−0.3449	0.06784	0.2121	0.1827	0.4885	0.4077	0.4398	0.4616	0.4069	0.3465	0.1634	−0.0415	0.2817	0.1939	0.1852
Cystine	0.7199	0.4966	0.5363	0.7	1	−0.3206	0.6468	0.07136	0.4800	0.4032	0.6438	0.7375	0.6883	0.7261	0.7259	0.5611	0.6098	0.4947	0.4326	0.6458	0.4638	0.6411
Gly	−0.6112	0.5425	0.5406	−0.1681	−0.3206	1	0.5394	0.7472	0.813	0.7472	0.7533	0.7203	0.8113	0.7755	0.576	0.576	0.4662	0.5104	0.3150	0.8100	0.8161	0.7563
Asn	0.222	0.708	0.9549	0.5277	0.6468	0.5394	1	0.5394	0.813	0.7472	0.6066	0.7203	0.8113	0.7755	0.7175	0.576	0.4662	0.5104	0.3150	0.8100	0.8161	0.7563
Ala	0.05874	0.7977	0.5231	−0.3449	0.0713	0.7472	0.5394	1	0.8801	0.8117	0.7838	0.5407	0.675	0.5518	0.5633	0.5332	0.4902	0.6947	0.6783	0.6783	0.7994	0.7852
Gln	0.3203	0.9113	0.7175	0.06784	0.4800	0.813	0.8130	0.8801	1	0.8869	0.9532	0.7925	0.8758	0.8577	0.8069	0.7126	0.6577	0.7586	0.6829	0.9545	0.9653	0.9545
Ser	−0.0009	0.9763	0.7515	0.2121	0.4032	0.7472	0.7472	0.8117	0.8869	1	0.8912	0.88	0.9402	0.8084	0.8873	0.8877	0.8139	0.9023	0.7692	0.9005	0.9108	0.8947
Val	0.4465	0.9527	0.6542	0.1827	0.6438	0.7533	0.6066	0.7838	0.9532	0.8912	1	0.9127	0.9474	0.9177	0.9232	0.837	0.8301	0.8805	0.829	0.9824	0.9232	1.0000
Leu	0.3242	0.9316	0.6542	0.4885	0.7375	0.7203	0.7203	0.5407	0.7925	0.8800	0.9127	1	0.9710	0.9338	0.9995	0.9636	0.9471	0.9112	0.8036	0.9273	0.8388	0.9139
Thr	0.2758	0.9593	0.7771	0.4077	0.6883	0.8113	0.8113	0.675	0.8758	0.9402	0.9474	0.971	1	0.9006	0.9729	0.914	0.8818	0.9124	0.7931	0.9483	0.8825	0.9489
Trp	0.4372	0.8843	0.6225	0.4398	0.7261	0.7755	0.7755	0.5518	0.8577	0.8084	0.9177	0.9338	0.9006	1	0.9376	0.8645	0.8231	0.7667	0.6646	0.9657	0.9171	0.919
Ile	0.3284	0.9407	0.6481	0.4616	0.7259	0.5760	0.7175	0.5633	0.8069	0.8873	0.9232	0.9995	0.9729	0.9376	1	0.9643	0.9487	0.9181	0.8154	0.9357	0.8501	0.9244
Glu	0.1306	0.9275	0.5365	0.4069	0.5611	0.576	0.576	0.5332	0.7126	0.8877	0.837	0.9636	0.914	0.8645	0.9643	1	0.9743	0.9362	0.8304	0.8574	0.7962	0.8394
Met	0.2678	0.8868	0.4191	0.3465	0.6098	0.4662	0.4662	0.4902	0.6577	0.8139	0.8301	0.9471	0.8818	0.8231	0.9487	0.9743	1	0.9544	0.9042	0.8169	0.7086	0.8305
Asp	0.1887	0.9423	0.5066	0.1634	0.4947	0.5104	0.5104	0.6947	0.7586	0.9023	0.8805	0.9112	0.9124	0.7667	0.9181	0.9362	0.9544	1	0.957	0.8384	0.7576	0.881
Tyr	0.3201	0.85	0.2882	−0.04157	0.4326	0.3150	0.3150	0.6783	0.6829	0.7692	0.829	0.8036	0.7931	0.6646	0.8154	0.8304	0.9042	0.957	1	0.748	0.6401	0.8273
Phe	0.3869	0.9530	0.6949	0.2817	0.6458	0.8100	0.8100	0.6783	0.9545	0.9005	0.9824	0.9273	0.9483	0.9657	0.9357	0.8574	0.8169	0.8384	0.748	1	0.9662	0.9836
Pro	0.208	0.9307	0.7082	0.1939	0.4638	0.8161	0.8161	0.7994	0.9653	0.9108	0.9232	0.8388	0.8825	0.9171	0.8501	0.7962	0.7086	0.7576	0.6401	0.9662	1	0.9263
Hyp	0.4394	0.9548	0.658	0.1852	0.6411	0.7563	0.7563	0.7852	0.9545	0.8947	1	0.9139	0.9489	0.919	0.9244	0.8394	0.8305	0.8810	0.8273	0.9836	0.9263	1

Arg—Arginine; Lys—Lysine; His—Histidine; Cys—Cysteine; Cystine; Gly—Glycine; Asn—Asparagine; Ala—Alanine; Gln—Glutamine; Ser—Serine; Val—Valine; Leu—Leucine; Thr—Threonine; Trp—Tryptophan; Ile—Isoleucine; Glu—Glutamic acid; Met—Methionine; Asp—Aspartic acid; Tyr—Tyrosine; Phe—Phenylalanine; Pro—Proline; Hyp—4-Hydroxyproline. V1—Endozym Thiol®, AEB; V2—Endozym β-Split®, AEB; V3—Zymovarietal aroma G®, SODINAL; V4—Endozym Ice®, AEB; V5—Zimarom®, BSG WINE; V6—control sample, no enzymes.

Arginine is one of the most important nitrogen compounds in wine [9]. Concerning Fetească regală variety, arginine consumption decreased in the second part of the fermentation process. According to Zhou et al. [16], this phenomenon can be explained by the action of protocatechuic and gallic acids (both not analysed in this experiment) that inhibit the enzyme arginine deiminase. V2 (Endozym® β-Split, AEB, San Polo, Italy) and V4 (Endozym® Ice, AEB, San Polo, Italy) showed the same trend, while in the other variants, different variations were dependent on the type of administered enzyme. In Fetească regală wines, the final concentration ranged from 400.27 mg/L in V1 (Endozym Thiol®, AEB, San Polo, Italy) to 180.50 mg/L (3 times lower) in V3 (Zymovarietal® aroma G, SODINAL, Plovdiv, Bulgaria). Its concentration in V6 (control sample) was about 282 mg/L. For the majority of Sauvignon blanc samples, arginine showed the same tendency in V1, V2 and V5 (Zimarom®, BSG WINE, Napa, California) variants, as they were consumed by yeasts in the first part of the fermentation phase, with a maximum concentration in the middle of the process and a slight decrease towards the end. In V4 and V6 samples, its concentration increased in all alcoholic fermentation stages. For Sauvignon blanc wines, the highest concentration in arginine was registered in V4 variant (18.75 mg/L), followed by V5 (18.32 mg/L), and the lowest value was identified in V1 sample (14.67 mg/L), while the control sample registered about 17.76 mg/L. The results highlight that important concentrations of this compound also resulted from the yeasts' metabolism.

Threonine results from the synthesis of aspartic acid [2]. Its degradation pathway is related to glycine synthesis [4]. Threonine content evolved similarly for the V2, V3, V4 and V6 Fetească regală varieties. The obtained wines showed minor differences between the analysed variants, ranging from 9.72 mg/L in V4 to 8.20 mg/L in the V1 variant. A positive correlation was found between threonine and aspartic acid. For the second category of samples, V2, V4 and V6 variants presented the same tendency. Important quantities were identified in Sauvignon blanc wines, with the highest level being recorded in V5 (15.00 mg/L), while the lowest value was highlighted in the V6 (11.47 mg/L) variant.

Aspartic acid can be synthesized by yeasts, from the amination reaction of succinic acid with ammoniacal nitrogen or from the enzymatic transamination of glutamic acid with succinic acid [2]. Most Fetească regală samples showed the same tendency in the evolution of aspartic acid, except for the V1 variant. Thus, significant quantities were assimilated by bacteria and yeasts in the first phase of the fermentation, and after the middle of the biochemical process, its levels significantly increased. Final concentrations of this compound ranged from 29.43 mg/L (V6) to 18.15 mg/L (V3) in Fetească regală wines. Aspartic acid showed the same tendency in most Sauvignon blanc variants; except for V3, significant amounts were accumulated in the second phase of the fermentation. The resulted wines were characterised by considerable concentrations of aspartic acid, which ranged from 45.38 mg/L in the V5 variant to 30.63 mg/L in the V6 sample.

Asparagine constitutes an amide of aspartic acid [2]. Similar to glycine, Fetească regală samples showed the same tendency of asparagine content in V2, V3 and V6 variants, with an initial decrease in the value during the first phase of the fermentation. According to Cusano et al. [16] it can be postulated that yeast metabolism is responsible for transforming these compounds' patterns and maintaining the redox potential of the cell. An important accumulation can be observed after the middle of the biochemical process. On the other hand, this compound showed the same tendency in V4 and V5 samples, with its concentrations constantly increasing. Thus, the obtained wines presented significant levels of asparagine in the V6 variant (55.06 mg/L) and the smallest value was obtained in V1 (36.41 mg/L). A positive correlation between asparagine and alanine (r = 0.8767) can be observed in Table 3.

For the Sauvignon blanc samples, the asparagine content also fluctuates depending on the type of applied enzymes. This compound was found in different amounts in wines, with the highest concentration being recorded in V2 (4.36 mg/L), followed by V5 (3.85 mg/L), and the lowest value was obtained in the V6 variant (2.97 mg/L). For this group of samples, a high positive correlation was registered between arginine and histidine

(r = 0.9549), glutamine (r = 0.8130), threonine (r = 0.8113), phenylalanine (r = 0.8100) and proline (r = 0.8161).

Serine usually results from glycocol, using an enzymatic pathway [2]. The compound showed the same tendency in most Fetească regală aliquots, except for the V1 variant. Thus, although at the beginning of the fermentation period, its level decreased, successive accumulations followed until the end of the fermentation stage, reaching up to 19.08 mg/L in V6 wine > 16.39 mg/L in V4 > 15.04 mg/L in V1 > 13.94 mg/L in V5 > 13.32 mg/L in V3 > 10.99 mg/L in V2. In Sauvignon blanc samples, considerable amounts of this compound were identified after alcoholic fermentation (30.37 mg/L in V3 > 30.07 mg/L in V5 > 29.58 mg/L in V2 > 25.44 mg/L in V4 > 23.60 mg/L in V1 > 22.04 mg/L in V6). Regarding Table 4, a linear relation between serine and lysine (r = 0.9763), threonine (r = 0.9402), or proline (r = 0.9108) can be noted.

Most of the lysine content may result from yeast metabolism [17] but is not preferred as a nitrogen source by yeast *Saccharomyces cerevisiae* spp. [4]. These compound concentrations presented the same trend (decreasing and then increasing the content after the middle of alcoholic fermentation) in most of the Fetească regală samples, except for V1 and V5 variants. After fermentation stage, V4 (14.93 mg/L) showed the highest value, followed by V1 (13.76 mg/L), while the lowest level of lysine was identified in V5 (7.33 mg/L). Lysine was consumed by yeasts and bacteria in the first part of the fermentation in Sauvignon blanc samples, and its level increased towards the end of the process. Thus, the lysine level in the resultant wines ranged from 10.03 mg/L in the V3 variant to 5.83 mg/L in V1.

Leucine is an essential amino acid [17,18], found in significant quantities in all experimental samples. Pyruvate is the source of leucine synthesis [2]. In wine, leucine precursors contribute to the formation of fruity aromas of nuts (2-methylpropanal), bananas and pears (acetic acid, isopentyl esters), honey, sweet taste, lactate (3-methylbutanoic acid) and the formation of fusel flavours (3-methylbutan-1-ol) [19]. Similar to the serine, valine and leucine showed the same tendency during the alcoholic fermentation of Fetească regală samples. V6 b the highest concentration (11.66 mg/L) of this compound, while V2 had the lowest content (obtained.27 mg/L). Very high linear correlations were obtained between leucine and tryptophan (with r > 0.9628). Leucine also followed the same evolution direction in all Sauvignon blanc variants. Thus, although significant amounts of this compound were consumed at the beginning of the alcoholic fermentation, important concentrations were accumulated in the second part of the process. McKinnon [4] reported a positive correlation between ethyl octanoate formation and leucine levels. Regarding the final concentrations, the leucine level varied from 19.23 mg/L in the V2 variant to 13.51 mg/L in V6.

Isoleucine, leucine and valine are precursors of isobutyl, amyl and isoamyl alcohols [2], which are responsible for fruity notes in wines. Isoleucine showed successive increases throughout the alcoholic fermentation period in most Fetească regală samples, except for the V1 variant, where its level decreased towards the end of the fermentation phase. The highest concentration was identified in the V1 variant (13.09 mg/L) and the smallest value can be observed in V3 sample (5.89 mg/L), about half of the level of the first-mentioned variant. The isoleucine concentration in Sauvignon blanc samples tended to successively increase with the evolution of alcoholic fermentation. Thus, the final content varied from 14.22 mg/L in V2 variant to 10.45 mg/L in V6. This phenomenon can be explained by the lactic bacteria activity, indicating the installation of malolactic fermentation. The increase in isoleucine concentration may be also correlated with the activity of some other bacteria, with the presence of threonine precursors—α-ketobutyric acid and α-amino butyric acid. The presence of L-homoserine in the medium, a *Saccharomyces cerevisae* and *Escherichia coli* metabolite, is favourable for the increase in isoleucine concentration [2].

Methionine presents an important antioxidant capacity, with an essential function in metabolic pathways. This compound is not consumed in low amounts by yeasts during fermentation but is indispensable to the development of lactic acid bacteria [2]. In Fetească regală samples, this amino acid followed the same tendency in V2–V3 variants, as well as V4–V6. After alcoholic fermentation, relatively low concentrations of methionine were

registered, ranging from 0.98 mg/L in V1 to 0.46 mg/L in V3. For Sauvignon blanc, V1, V3 and V4 variants indicated the same trend. After the alcoholic fermentation, methionine ranged from 1.33 mg/L in V3 and V4 variants, followed by 1.29 mg/L in the V5 sample, 1.25 mg/L in V2, 1.03 mg/L in V1 and 0.99 mg/L in V6, respectively. A linear relation was found between methionine valine, phenylalanine and 4-hydroxiproline, regardless of variety.

Glutamine is a precursor of asparagine and tryptophan and the amide of glutamic acid [4]. For the Fetească regală variety, this compound followed a decreasing tendency in V2, V3 and V4 variants, and was a great nitrogen source for yeasts [20]. V1 sample showed for the highest value for glutamine (20.78 mg/L). Positive correlations were obtained between glutamine vs. alanine, valine, lysine and methionine, as shown in Table 3.

Regarding the Sauvignon blanc variety, an upward evolution was observed during the fermentation stage for the majority of the experimental samples. This compound can be converted from glutamate, which is produced by ammonium assimilation and amino acids transamination reactions [21]. The V5 sample presented the highest concentration of glutamine (17.77 mg/L), followed by V2 (with 17.27 mg/L), while the lowest value was registered in V1 (11.07 mg/L). A linear correlation was found between glutamine and lysine (r = 0.9113), phenylalanine (r = 0.9545), proline (0.9653) and 4-hydroxyproline (r = 0.9545) (Table 4).

Proline is the most abundant amino acid in wine [4]. Its values were ascendant during alcoholic fermentation for the majority of the Fetească regală variants. McKinnon [4] suggested that proline results from arginine degradation. The resulting wines were characterised by considerable levels of proline (from 429.08 mg/L in V2 to 338.06 mg/L in V1). Even if this compound is not consumed by the yeasts during fermentation, most Sauvignon blanc samples showed successive decreases in proline concentrations. This phenomenon can be explained by the oxidative metabolism of yeast on nitrogen compounds, including proline [4]. In the first stage of alcoholic fermentation, important concentrations of this compound can be assimilated due to the partially aerobic conditions [4]. For the Sauvignon blanc wines, the following concentrations were obtained: V2 (321.85 mg/L) > V3 (307.06 mg/L) > V5 (303.37 mg/L) > V4 (260.3 mg)/L) > V6 (250.75 mg/L) > V1 (224.07 mg/L).

4-Hydroxyproline represents a proline-derived compound and is not generally metabolised by *Saccharomyces cerevisiae* in classical winemaking conditions [2]. Its values presented different fluctuations during the fermentation stage. The content of Fetească regală wines in this amino acid varied from 3.38 mg/L in V1, to 2.44 mg/L in V3. From Table 3, a positive correlation can be observed between 4-hydroxyproline and isoleucine, tyrosine and proline can, with their values being dependent on one another.

For Sauvignon blanc samples, the maximum concentration was obtained at the middle of the fermentation stage for V1, V2 and V6, followed by an important reduction towards the end of the process, in contrast with V3 and V5 variants. Final concentrations of 4-hydroxyproline ranged from 6.11 mg/L in V5 to 4.33 mg/L in the V1 sample.

Alanine is one of the most important nitrogen sources for yeasts and usually results from pyruvic acid, either after the decarboxylation of aspartic acid or through the transamination reaction or results after the involvement of ammoniacal nitrogen [2,17]. V3–V6 and V4–V5 groups showed a similar trend regarding alanine evolution in Fetească regală samples. After alcoholic fermentation, the highest concentration was identified in the V1 sample (119.30 mg/L), followed by V2 (60.35 mg/L), V4 (44.96 mg/L), V6 (42.84 mg/L), V3 (36.17 mg/L) and lastly, V5 (29.89 mg/L), which was four times lower than the first-mentioned variant. The very high correlations between alanine and glycine (r = 0.9749) and valine (r = 0.9354) are highlighted in Table 3. The content of Sauvignon blanc samples in alanine simultaneously increased with the sampling moment in most variants, except V1 and V4. Regarding the final alanine levels in wines, values between 55.74 mg/L (V5) and 41.65 mg/L (V1) were recorded.

Regarding cysteine, an amino acid with an -SH group, which results from the reaction of serine with inorganic sulphur [2], V1 and V2 samples presented the same tendency, which was similar to that of V1 and V3 for cystine case, a dimer of cysteine. The formation of cystine from cysteine is a reversible process, favoured by the presence of metallic ions [2]. This compound is usually found in reduced concentrations in the must (below 10 mg/L). Considering their catabolic network, these amino acids can be considered important precursors for the synthesis of some volatile sulphur compounds [22]. In Fetească regală samples, the final concentrations of cysteine varied from 0.11 mg/L (V1, V3 and V5) to 0.04 mg/L (V2). The cystine levels were relatively low, ranging from 0.15 mg/L in V4 to 0.06 mg/L in V2 variant. Positive correlations can be observed between cystine and serine (r = 0.8588), leucine (r = 0.8312) and tryptophan (r = 0.8332) in Table 3. Histidine, cysteine and cystine were not identified in samples in the V4, V5, and V6 variants. Regarding the evolution of cysteine in Sauvignon blanc samples, important quantities of this compound were consumed during the first stage of alcoholic fermentation while significant increases were registered in the second stage for V2 and V5 variants. For the rest of the samples, an initial reduction was recorded, with important quantities being accumulated towards the middle of the fermentation. Significant amounts are consumed at the final stage of the biochemical process. The V2 sample presented the highest value of this compound (0.14 mg/L), followed by V4 (0.11 mg/L). Cysteine was almost completely assimilated in Sauvignon blanc V6 variant (0.01 mg/L). The cystine content showed a significant reduction in the second phase of alcoholic fermentation in most samples, except the V4 variant. Final concentrations in cystine varied from 0.03 mg/L in V1, V3 and V6, to 0.07 mg/L in V2 and V4. Arginine, lysine, histidine, cysteine and cystine were not identified in the V3 variant.

Glycine, along with ethanol, residual sugar, and glycerol, contributes to the sweet taste [17] and has not been reported as a good source of nitrogen for *Saccharomyces* spp. [23]. Its concentration values decreased after the first stage of the fermentation in V1 and V2 Fetească regală samples. To explain this phenomenon, Scott et al. [24] reported a positive correlation between glycine level and fusel alcohols and acetate esters in wine. The decrease in concentration during fermentation may be due to deamination reactions and the fact that amino acids actively participate in numerous chemical reactions [23]. Important quantities were accumulated at the end of the process, possibly resulting from serine [25]. After the fermentation phase, the highest amount of glycine was identified in the V1 variant (13.60 mg/L), while the lowest value was recorded in the V5 sample (3.74 mg/L). Several positive correlations were generated by Pearson analysis between glycine and alanine, leucine, tyrosine, 4-hydroxyproline etc. (Table 3). For the Sauvignon blanc variety, glycine levels presented an upward evolution for the majority of the variants. The amount of glycine in Sauvignon blanc ranged from 12.88 mg/L in V3 to 7.60 mg/L in V4.

Valine is generally produced by the amination of pyruvic acid [2], and is a precursor of apple flavour (2-methylpropanal, 2-methylpropanoic acid) and fruity notes (2-methylpropan-1-ol), banana (2-methylpropyl acetate). Krogerus and Gibson [26] reported a negative correlation between valine and total diacetyl and 2,3-pentanedione levels during wine fermentation. Grapes harvested at over-ripeness may present high amounts of this compound [17]. In the first sample category, valine showed the highest levels in V1 wine (12.70 mg/L) and the lowest value was registered in V5 (7.82 mg/L). For the Sauvignon blanc samples, this value presented successive increases in V3, V5 and V6 samples, resulting from chemical reactions. For the rest of the samples, significant quantities were consumed by the yeasts during the first part of the fermentation process, with important concentrations being accumulated in the second stage of wine formation. After alcoholic fermentation, the V5 variant showed the highest level (14.62 mg/L), while the lowest value was obtained in V1 (10.25 mg/L).

Phenylalanine, the precursor of phenyl ethyl acid (imprints the aroma of honey to wine) [2], showed an initial reduction in concentration and consequent increases after the middle of the alcoholic fermentation. Thus, wines' concentrations in these compounds varied from 17.05 mg/L in the V1 variant to 10.75 mg/L in V2. Similar to tyrosine, pheny-

lalanine showed the same tendency for all Sauvignon blanc variants. The two mentioned amino acids participated in the formation of phenolic compounds [23]. Significant quantities were assimilated by yeasts during the alcoholic fermentation to form higher alcohols (Ehrlich pathway) [20], but important quantities were accumulated in the second stage of the process. The resulted wines were characterised by their high phenylalanine contents, with values varying from 27.67 mg/L in V2 to 17.61 mg/L in V3 variant.

Tyrosine results from phenylalanine (by 4-hydroxylation) [17] and is the precursor of tyrosol. It is generally consumed by lactic acid bacteria. Most Fetească regală samples showed the same tendency in tyrosine evolution, with successive increases in concentration after the second stage of fermentation. The highest value of tyrosine was identified in V1 sample (6.13 mg/L), followed by V6 (4.18 mg/L), V4 (3.63 mg/L), V2 (3.50 mg/L), V5 (3.01 mg/L) and finally, V3 (2.65 mg/L). Tyrosine is positively correlated with tryptophan and isoleucine (Table 3). Regarding the Sauvignon blanc variety, tyrosine content presented a significant diminution of the initial concentrations during the first stage of fermentation, which could be explained by the yeasts' consumption of amino acid. However, an important accumulation of tyrosine can be observed after the middle of the process, which influenced by the type of administered enzyme. Regarding the content of the obtained wines in tyrosine, the highest value was recorded in the V5 sample (9.30 mg/L), followed by V3 (8.93 mg/L), V4 (8.60 mg/L), V2 (7.60 mg/L), V6 (6.78 mg/L) and finally, V1 (6.61 mg/L).

Tryptophan, the precursor of B_3 vitamin, is one of the most assimilable amino acids by bacteria and yeasts [2]. Analysing the Fetească regală experimental variants, the same tendency can be observed in V2, V3 and V6 samples. Final concentrations varied from 1.70 mg/L in V1 to 0.82 mg/L in V3. For the Sauvignon blanc variety, this amino acid showed the same trend in V1, V3 and V4 samples (Figure 3). The highest concentration of tryptophan can be observed in V2 wine (6.12 mg/L), followed by V3 and V5 (5.17 mg/L). The lowest concentration was identified in the V1 sample (2.86 mg/L), which was approximately three times lower than that found for the first. From Pearson analysis, tryptophan values are dependent on leucine values for both varieties.

Histidine biosynthesis in grapes is strongly connected with nucleotide biosynthesis. Histidine can act as a precursor for tryptophan. Glutamic acid and glutamine act as nitrogen donors in histidine biosynthesis [27]. Histidine concentration showed different variations, depending on the type of applied enzyme and fermentation stage. Its levels in Fetească regală wines varied from 55.71 mg/L in V1 to 6.85 mg/L in the V3 variant. The evolution of histidine followed the same direction in most Sauvignon blanc samples, with important quantities being consumed during the first fermentation stage and significant increases towards the end of the process. According to López-Rituerto et al. [28], histidine is converted into histaminol in the first phase of the biochemical process, when ethanol content is close to 5% vol. For Sauvignon blanc wines, the V2 variant presented the highest value (0.14 mg/L), with the lowest level being recorded in the V6 sample (0.01 mg/L).

Glutamic acid is essential in the formation of succinic acid and presents a sweet taste [17]. For the first category of samples, glutamic acid evolved in the same direction for the V1, V3 and V5 variants. Fetească regală wines showed the lowest level of glutamic acid in the V3 variant (18.35 mg/L), while the highest value was presented by the V1 sample (30.27 mg/L). Regarding the Sauvignon blanc samples, glutamic acid showed the same tendency in V1–V3 and V5–V6 samples, respectively. The experimental wines were distinguished by the high amounts of this compound, with the highest value being registered in the V3 variant (41.26 mg/L), and the lowest content of glutamic acid was obtained in V6 (30.29 mg/L). A linear correlation was obtained between glutamic acid and valine, phenylalanine and 4-hydroxyproline for both varieties.

The enzymatic treatments determined significant differences between the concentrations of the main identified amino acids ($p < 0.05$). According to Fisher's test, the obtained averages showed significant differences between most pairs of variables.

In general, Fetească regală samples were characterised by having the highest concentrations of amino acids. However, according to the results, cysteine, cistine and glycine

contents were not influenced by variety, with their levels being similar among the analysed varieties. The highest concentrations for the majority of compounds can be observed in V1 for Fetească regală samples and V2 for Sauvignon blanc. High arginine and alanine contents were found in the experimental Fetească regală wines. Some authors [10,29] obtained considerable arginine concentrations in wine. Similar to the results presented by Cosme et al. [30], arginine, alanine, aspartic acid and glutamic acid were the main identified nitrogen compounds when proline is not included. Álvarez-Fernández [31] postulated that arginine, aspartic acid, leucine, isoleucine, methionine, lysine, serine, threonine and asparagine are preferential sources for *Saccharomyces cerevisiae* yeasts. The amino acids with phenyl radical (phenylalanine, tyrosine and tryptophan) can also be consumed, but they are less preferable [31]. According to Valero et al. [32], both proline and arginine are not consumed by yeasts during alcoholic fermentation due to the anaerobic conditions. L-proline is found in higher quantities in wine than in the initial must due to nitrogen catabolite's inhibition of proline permease and the limited supply of molecular oxygen that is essential for proline oxidase activity [32]. Moreover, arginine inhibits proline utilisation in yeasts [33]. After the end of the fermentation process, the yeasts develop an oxidative metabolism regarding wine's carbon compounds (such as ethanol or glycerol) or other nitrogen components (including L-proline). During the maturation phase, proline could be the principal source of nitrogen [32], explaining why, in some Sauvignon blanc samples (V1, V3), proline levels descended after the end of the fermentation. Many positive correlations (with values that are proportional one to the other) between arginine and different compounds were obtained, such as lysine, leucine, isoleucine, methionine, tyrosine, etc.

As described by Castor [34], changes in the amount of every analysed amino acid correlated with the two phases of the microorganism's evolution (not monitored in this work). The decrease in most amino acids correlates with the yeasts' multiplication. Glutamic acid, glycine, valine and glutamine decreased during the first phase of the fermentation of Fetească regală samples, while for the Sauvignon blanc the same tendency was noticed for alanine, serine, arginine, lysine, glutamic acid and lysine. As the cell number is relatively unmodified, the second phase of the fermentation correlated with the increasing concentration of methionine, tyrosine, lysine, cysteine and phenylalanine, independently of the studied varieties. *Saccharomyces cerevisiae* yeast is able to deposit amino acids in the vacuole [32]. According to Castor [34], autolysis could justify the increasing concentrations of amino acids in the fermenting must.

Pogorzelski et al. [35] postulated that when the levels of arginine, phenylalanine, serine, isoleucine, histidine and methionine are diminishing, alanine, glycine, and lysine concentrations are ascendant. In this work, this affirmation is confirmed in some cases. Therefore, when arginine and serine decrease, amino acids such as alanine, glycine and lysine increase for both varieties.

According to the results, both pectinases and yeast activity provide the most effective results, while V1 sample (Endozym Thiol®, AEB, San Polo, Italy) registered the highest values for the most identified compounds, especially essential amino acid (histidine, isoleucine, methionine, phenylalanine, tryptophan and valine). After V1, the control variant (V6) recorded the highest proportions of asparagine, serine, leucine and aspartic acid. The results highlighted that the enzymes administrated in V2, V3, V4 and V5 variants generated a concentration decrease for the mentioned amino acids. The lowest values for most of the identified compounds were obtained for variant V3. Sauvignon blanc wines have been noted for their high proline, alanine, glutamic acid, aspartic acid and serine contents. Beltran et al. [36] reported comparable amounts of asparagine (approximately 45 mg/L), lysine (16 mg/L) and proline (approximately 500 mg/L). Guitard et al. [37] presented a range from 870 to 1770 mg/L proline in Chardonnay must. Bell and Henschke [38] reported 27–454 mg/L glutamic acid, 10–138 mg/L aspartic acid and 13–330 mg/L serine in grape juice. Following the observations reported by the literature, comparable amounts of the main amino acids were registered in the analysed samples. For this category of samples,

V2 (Endozym® β-Split, AEB, San Polo, Italy) presented the highest concentrations in most of the identified compounds.

The results indicate an important variation in the amino acid profile depending on the applied enzymes and the grape varieties. While the analysed samples are obtained by grapes from the same vineyard and the same agricultural practices are applied, the data suggest that the differences between samples are a consequence of the intrinsic characteristics of both grape varieties under the influence of the applied treatment.

3.2. The Impact of Enzymes on Wines Sensory Perception

The experimental samples presented different sensory properties depending on the administrated enzymes and analysed variety (Figure 4). Fetească regală wines were defined by an intense fruity aroma (exotic fruits, ripe fruits, dried fruits) and wildflower notes. Regarding the sensory descriptors that were less appreciated by consumers (for example, the phenolic and bitter sensation), the lowest intensity was registered in the V1 sample. In general, all samples treated with enzymes showed lower values for the vegetable and mineral character of Fetească regală wines. Sauvignon blanc wines have been described as more vegetal and spicy, with mown hay notes, minerals, and an intense citrus and exotic fruits aromas. For these samples, the bitter sensation was the most pronounced in the V2 variant. In accordance with the obtained results, an intense honey taste was noted in variants with considerable leucine and phenylalanine proportions, with the second compound being the precursor of phenyl ethyl acid [2]. As seen in Figure 4, samples with higher concentrations of valine, leucine and isoleucine were characterized by their intense fruity notes, probably explained by the fact that these three compounds are precursors of isobutyl, amyl and isoamyl alcohols [2]. According to Figure 4, the higher intensity of the sweet taste in V1—Fetească regală—and V3—Sauvignon blanc—is related to their glutamic acid and glycine proportions.

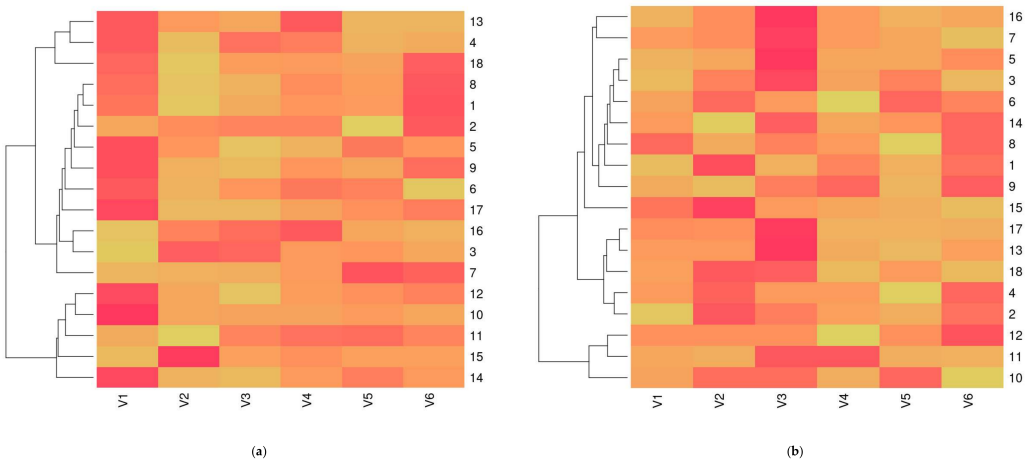

Figure 4. Sensory perception of resulted wines: (**a**)—Fetească regală; (**b**)—Sauvignon blanc. **1**—vegetable; **2**—mineral; **3**—citrus, **4**—ripe fruit; **5**—exotic fruits; **6**—dried fruits; **7**—green fruits; **8**—mown hay; **9**—wildflowers; **10**—rose; **11**—spices; **12**—honey; **13**—acid; **14**—sweet; **15**—bitter; **16**—phenolic; **17**—unctuous; **18**—texture. **V1**—Endozym Thiol®, AEB; **V2**—Endozym β-Split®, AEB; **V3**—Zymovarietal aroma G®, SODINAL; **V4**—Endozym Ice®, AEB; **V5**—Zimarom®, BSG WINE; **V6**—control sample, no enzymes.

Moreover, samples with strong sweet notes usually presented less of a bitter taste. This phenomenon can be explained by the action of glutamic acid, which is known to affect the perception of other tastes: when sweetness is enhanced, bitterness is diminished [39]. According to the obtained results, wine with higher residual sugar presents considerable amounts of glutamic acid.

In accordance with sensory evaluation, Bakker et al. [40] also reported a significant increase in the intensity of positive sensory descriptors in samples treated with pectinases

compared to the control sample. McKinnon [4] reported a positive correlation between fruity (especially exotic fruits) and floral notes and leucine levels. Contrary to the results obtained for the Fetească regală variety, the lowest content of most amino acids was found in V1 and V6, highlighting the variability of these varieties. Numerous authors have monitored the level of nitrogen compounds and their variation during the winemaking process [41,42]. Some amino acids, such as tyrosine, glycine or arginine, were not consumed by *Saccharomyces cerevisiae* yeasts in Sauvignon blanc samples, which confirms previous observations made on white wines by Pinu et al. [42]. The results may differ depending on the variability of the variety and the type of administered enzyme. Thus, the supplementation with pectinases was effective in the Fetească regală samples, although the β-glycosidases showed the highest values in the Sauvignon blanc wines. According to Ugliano et al. [43], a high-intensity confectionary taste or red fruit flavor were obtained in Shiraz wines when nitrogen was supplemented before alcoholic fermentation in must. Additionally, the intensity of negative descriptors such as earth, cheese and yeast was suppressed. Hernández-Orte et al. [44] highlighted more citric and less sulphurous odour in Airen musts when high levels of nitrogen compounds were present.

The efficiency of the enzymes is also influenced by the stage of enzyme administration. Thus, in the case of Fetească regală wines, the most effective were samples in which the enzymes complied with the manufacturer's administration recommendations. However, it has been shown that β-glycosides can produce effective results in increasing amino acids concentrations in Sauvignon blanc wines when administered at the beginning of the fermentation in must, even if the producers recommend that they are administered at the end of the biochemical process.

This paper contributes to the enrichment and consolidation of the specialised literature regarding the influence of various enzyme preparations on amino acid content, increasing the assortment of wines and optimisation of winemaking technology at both laboratory and industrial scales. Enzymes can enhance the amino acid proportions in wines with minimal techniques and low levels of energy consumption. Both pectinases and yeast activity can lead to effective results regarding the amino acid content, especially essential ones (histidine, isoleucine, methionine, phenylalanine, tryptophan and valine).

The efficiency of the enzymes is also influenced by the stage of enzyme administration. Thus, in the case of Fetească regală wines, the most effective at increasing amino acid levels were samples in which the enzymes complied with the manufacturer's administration recommendations. However, it has been shown that β-glycosides contribute to the higher content of the main amino acids found in Sauvignon blanc wines when they are administered at the beginning of the fermentation in must, even if the producers recommend their administration at the end of the fermentation stage. Pectinases can provide more acceptable results regarding the sensory perception of wines, with these variants being noted for their fruity notes.

4. Conclusions

The amino acid profile of the analysed samples was influenced by the type of supplemented enzymes, grape variety and the stage of administration. Both pectinases and yeast activity can lead to favourable results regarding the amino acid content, especially essential ones (histidine, isoleucine, methionine, phenylalanine, tryptophan and valine). Supplementation with pectinases was effective in the Fetească regală samples, with these wines being noted for their significant contents of the majority of amino acids. The control sample presented the highest proportions of asparagine, serine, leucine and aspartic acid, highlighting that the considerable amounts of those compounds resulted from yeast metabolism under the action of endogenous enzymes. The wines treated with pectinases were defined by an intense fruity aroma, which was correlated with the significant contents of some amino acids, such as valine, leucine and isoleucine. β-glycosides enzymes preparations generated higher quantities of most amino acids in Sauvignon blanc wines. These

wines were defined by more vegetal, spicy, mineral and bitter notes, which were associated with their high concentration of glutamic acid.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12061406/s1, Table S1: Chromatographic working conditions.

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