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Araştırma Makalesi / Research Article

Modeling the Change in Amino Acid and Peptide Contents during the Aging of Bottled-Fermented Sparkling Wines by Image Processing Methods

Adnan BOZDOĞAN[1*], Ahmet CANBAŞ[2], Selahaddin Batuhan AKBEN[3]

Abstract

In this study, natural sparkling wine was produced using a mixture (1:1) of Emir and Dimrit grapes harvested (Around the Urgup District of Turkey). The change of free amino acid and peptide contents in sparkling wine depending on the aging time and yeasts (free and immobilized forms of the *Saccharomyces bayanus* and *Saccharomyces oviformis*) used in the second fermentation stage were examined by image processing methods. The immobilized yeasts were determined as more than the free ones along the second fermentation. It was also determined that the yeast type does not have a significant effect on both the peptides and free amino acid contents of sparkling wines and these contents were divided into 3 classes from high to low by measuring separately along the aging time. The amino acid contents of sparkling wines reached the highest level between the 335th and 365th days of aging time and also exceeded the amino acid contents of base wine.

Keywords: Natural sparkling wine, Amino acid and Peptide, Immobilized yeast, Image processing.

Doğal Köpüren Şarapların Yıllandırılması Sırasında Amino Asit ve Peptid İçeriklerindeki Değişimin Görüntü İşleme Yöntemleriyle Modellenmesi

Öz

Bu çalışmada, Türkiye'nin Ürgüp İlçe'sinden toplanan Emir ve Dimrit üzümleri karışımından (1:1) doğal köpüren şarap üretilmiştir. İkinci fermantasyon aşamasında kullanılan mayalara (*Saccharomyces bayanus* ve *Saccharomyces oviformis*'in serbest ve immobilize formları) ve ikinci fermantasyon süresine bağlı olarak Köpüren şaraptaki serbest aminoasit ve peptid içeriklerindeki değişim görüntü işleme yöntemleriyle incelenmiştir. İkinci fermantasyonda immobilize mayaların serbest mayalara göre daha fazla olduğu belirlenmiştir. Ayrıca, maya tipinin köpüren şarapların hem peptidleri hem de serbest amino asit içerikleri üzerinde önemli bir etkisinin olmadığı belirlenmiş ve bu içerikler yıllandırma süresi boyunca ayrı ayrı ölçülerek yüksekten düşüğe doğru 3 sınıfa ayrılmıştır. Köpüren şarapların aminoasit içerikleri yıllandırma süresinin 335. ile 365. günleri arasında en yüksek düzeye ulaşırken, temel şarabın aminoasit içeriğini de geçmiştir.

Anahtar Kelimeler: Doğal köpüren şarap, Amino asit ve Peptid, Tutuklanmış maya, Görüntü işleme.

[1]Osmaniye Korkut Ata University, Department of Food Engineering, Osmaniye, Türkiye, bozdogan@osmaniye.edu.tr
[2]Çukurova University, Department of Food Engineering, Adana, Türkiye, acanbas@cu.edu.tr
[3]Osmaniye Korkut Ata University, Department of Electrical and Electronics, Osmaniye, Türkiye, batuhanakben@osmaniye.edu.tr

[*]Sorumlu Yazar/Corresponding Author
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1. Introduction

The wines containing high amount of CO_2 are called as sparkling wine. These wines are produced by performing two consecutive fermentation processes. The characteristic wine is obtained at the end of the first fermentation then CO_2 is created in this wine by applying second fermentation. Thus, the ability to foam is also gained to the characteristic wine at the end of the second fermentation (Ribereau-Gayon et al., 2000; Martinelli et al. 2003). The most specific and known among sparkling wines is Champagne. It is produced using the mixture of Pinot noir or Pinot meunier black grapes and Chardonnay white grape that are grown in the Champagne region of France. Outside the Champagne region of France and in other countries, wines produced by the same method are also called Champagne and these wines are classified as the natural sparkling wine (Ribereau-Gayon et al., 2000). The second fermentation stage of champagne production is carried out in a bottle by adding yeast and sugar to the wine. Therefore, the amount and properties of the yeast used in the second fermentation are very important because the yeast to be used in the second fermentation should be able to work at low temperature, high CO_2 pressure and in a medium containing more than 10% alcohol, while it should be separated from the liquid in a short time after fermentation (Martinez-Rodriguez et al., 2001). After the second fermentation the wine is aged on the yeast lees for at least 1 year and the yeasts autolyses during the aging process. Thus, compounds (amino acids, peptides, proteins and polysaccharides, nucleic acids, enzymes etc.) that contribute positively to sensory properties are also obtained during autolysis (Feulliat and Charpentier, 1982; Martinez–Rodriguez et al., 2002). Among these compounds, amino acids and peptides are the most striking ones because they pass into the wine and positively affect the quality of the foaming wine by creating a thinner and permanent foam (Marchall et al., 1999; Martinez-Rodriguez et al., 2003; Culbert et al., 2017). After the second fermentation and aging processes completed, the yeast lees are removed from the bottle and the bottles are placed to the wooden racks in an inclined position and turned. Thus, the yeast lees are collected on the cork cover and removed. Various alternative methods are used for yeast precipitation, such as chemical additives, cartridge insertion, the use of flocculent yeast, and the use of immobilized yeast. Therefore, yeast precipitation takes several weeks and requires considerable labour (Ciani and Ferraro, 1998; Benucci et al., 2019).

As described above, the production of Champagne is a very laborious and attention requiring process, so the economic value of Champagne is also high. For this reason, researchers are working to produce quality Champagne using the appropriate methods and grapes grown in geographical regions characteristically like the Champagne region of France. Cappadocia region (Around the Urgup District of Nevsehir Province) in Turkey is similar to France's Champagne region as the geological structure, having underground cellars and widespread cultivation of grape. Also, the grape

variety named Emir grown in this region is suitable for the production of good quality white wine. In addition, the black grape variety named as Dimrit is widely grown in this region. Similarity of the Cappadocia region in Turkey and Champagne region in France suggests the grape varieties named Emir and Dimrit can be used instead of grape varieties named white Chardonnay, black Pinot noir and Pinot meunier which are claimed to give fullness to the taste of champagne.

Many studies for the suitability to wine of grape varieties grown in the Cappadocia region of Turkey are available in the literature (Cabaroğlu et al., 1997; Canbaş et al., 2001). In addition, studies were carried out on the production of natural sparkling wine using the grape variety named Emir and it has been determined that high quality natural sparkling wine can be produced from this grape (Bozdogan and Canbaş, 2011). Similar studies were also carried out for the grape variety named Dimrit and it has been determined that this grape variety is suitable for the production of natural sparkling wine of acceptable quality (Bozdogan and Canbaş, 2012). However, there is no study in the literature regarding the production of natural sparkling wine using a mixture of grape varieties named Emir and Dimrit. So, the effects of these varieties on amino acid and peptide contents of wine have also not yet been determined. Whereas it may be possible to produce a quality sparkling wine using the mixture of these grape varieties. For this reason, natural sparkling wine to be produced by traditional method using the mixture of varieties named Emir and Dimrit was studied in this study. Thus, the effects of the yeast strain used in the second fermentation and the aging time on the free amino acid and peptide contents were analysed. As a result, how to produce better quality natural sparkling wine using the mixture of grape varieties named Emir and Dimrit has been suggested.

For the visual and better analysis, the data measured in the experiments were transformed into images and image processing algorithms were used. In this way, the day interval of aging time was suggested instead of precise day that significant change occurred in and more tolerant and more resistant results were produced for future studies.

2. Materials and Methods

2.1. Materials

In the experiments, the varieties of white grape named Emir and black grape named Dimrit harvested in Cappadocia region (Around Urgup District of Nevsehir Province) of Turkey were used. The malt extract used to determine the total yeast number was obtained from Agar Merck (Germany). Alginic acid was obtained from Sigma (Germany). L-Aspartic acid, L-Glutamic acid, L-Asparagine, DL-Serine, L-Glutamine, L-Histidine, L-Threonine, L-Arginine, DL- Alanine, L-Tyrosine, γ- Amino butyric acid (GABA), Ethanolamine, L-Valine, DL-Methionine, DL-Tryptophan, L-Phenylalanine,

L-Isoleucine, L-Leucine and L-Lysine were obtained from Merck (USA). Methanol (HPLC grade) and Acetonitrile (HPLC grade) were purchased from Merck (Germany). All buffers and chemical solutions used in HPLC analysis were prepared with ultra-pure water (Obtained from Milli-Q Millipore device).

2.2. Arrangement of Trials

The trials were carried out in the Pilot Winery of the Çukurova University Food Engineering Department. In the production of naturally sparkling wines, the base wines obtained from a mixture of grape varieties named Emir and Dimrit in a ratio of 1: 1 were used. The fermentation in the bottle was carried out with free and immobilized yeasts. Measurement and application were carried out in three replications for each independent variable combination. Grapes were harvested after it was determined that they reached the appropriate maturity for sparkling wine production. The harvested grapes were transported to Pilot Winery in plastic cases. The grapes were squeezed in a horizontal press without being crushed then the obtained grape juice was grouped, and the juice group obtained with 50% yield as a result of the first squeezing was used for making quality champagne. The grape juice obtained was taken into the cellar at 15 ºC after sulfurized at a rate of 30 mg/kg and kept in the cellar for 24 hours to remove precipitated particles. At the end of this period, the precipitate was separated, and grape juice was fermented in a 250 L stainless steel tank in the cellar at 18 ºC to make its content alcoholic. Commercial S. *cerevisiae* yeast was added to the grape juice at the rate of 0.2 g/L before fermentation. The fermentation period was controlled by daily density and temperature measurements. After the fermentation was over, the wines were transferred to another fermentation tank and left for malolactic fermentation.

Malolactic fermentation was carried out with commercial lactic acid bacteria named as *Leuconostoc oenos*. Lactic acid bacteria were grown under sterile conditions and then added to wine at a rate of $1.8x10^6$ cells/mL by counting in Thoma slide. Malolactic fermentation period was controlled by paper chromatography method. After these stages, the wines were transferred to another tank then30 mg / L SO_2 was added to the wines and they left to rest in the cellar varying between 10-15 ºC. The second transfer of the wines was made and an additional 30 mg/L SO_2 was added.

The wines were then transferred again and an additional 30 mg/L SO_2 was added. In order to clarify the wines, a preliminary trial of clarification has been carried out with different ratios of various clarification agents (Gelatin, Gelatin + Tannin, Bentonite, PVVP and Casein). The best result was 24 g/hL casein for Emir and 42 g/hL for Dimrit. The casein determined as a result of the preliminary trial was added to the wines and clarification was carried out. The wines were then transferred to another tank and clarified by filtration. Finally, the wines obtained from Emir and

Dimrit grapes were mixed in a ratio of 1: 1. Thus, wine was obtained with a composition (1:1) of Emir + Dimrit grapes to use it for the second fermentation.

2.3. Preparation of Inoculation Culture

The used *S. bayanus* and *S. oviformis* yeasts were grown in malt extract agar. Two colonies of the *S. bayanus* and *S. oviformis* yeasts grown in accordance with the literature were inoculated into sterile wine containing 11º alcohol and 60 g/L sugar in an Erlenmeyer flask and the wines inoculated were mixed in an orbital mixer for 72 hours at 23-25 °C and 160 rpm (Yokotsuko et al., 1997). Thereafter, the wines were centrifuged in sterile tubes at 4000 rpm for 10 minutes. As a result of this process, sterile pure water was added to the yeasts separated from the wine and again centrifuged (Eppendorf Centrifuge 5810, Germany) at 4000 rpm for 10 minutes. Finally, the yeasts were counted using thoma slide and microscope, and inoculation was carried out at $2x10^6$ cells/mL per bottle.

2.4. Immobilization of Yeasts into Alginate Gel

Immobilization into alginate gel was done in accordance with the literature (Fumi et al., 1988; Ciani and Ferrora, 1998). Yeasts were immobilized in Na-alginate gel in immobilization trials. For this process, 2% Na-alginate and 0.5 M $CaCl_2$ solution to be used were sterilized first.

Yeasts used in immobilization were grown as described above and added to the Na-alginate solution under aseptic conditions. The prepared mixture was dropped into the sterile $CaCl_2$ solution at a flow rate of 2.5 mL/min from a height of 4-5 cm with the help of a peristaltic pump (Watson Marlov, England) so as to form beads with a diameter of approximately 2-3 mm or less. The beads were kept in the $CaCl_2$ solution for 2 hours to complete the gel formation. Then, the beads were separated from the $CaCl_2$ solution and washed with sterile distilled water, and 4.5 g of gel beads per $2x10^6$ cells / mL were added to the wine for each bottle (Pisinelli et al., 1989).

2.5. Second Fermentation, Aging and Residue Removal

Base wines obtained from the mixture of Emir + Dimrit (1: 1) were placed in 750 ml champagne bottles then sugar and yeast were added in order to gain enough CO_2 to create 4-6 atmospheres pressure. 24 g/L sugar was added to the wine in order to create 6 atmospheres pressure in addition to S. *bayanus* and *S. oviformis* yeasts to perform fermentation since 4 g/L sugar addition creates 1 atmosphere pressure in wine. Later, the bottles were closed with a cork and left for the second fermentation at 18 ºC.

During fermentation, the bottles were kept in a horizontal position. The sparkling wines were aged over the yeast following the second fermentation. After this process, the bottles were placed in special wooden racks after the yeast residue was dispersed in the wine by hand shaking. The bottles were initially placed in a horizontal position on the wooden racks the residue was then collected on the cork by moving bottles left and right every day and narrowing the angle between the wooden racks and the bottle.

In order to expel the residue from the bottle, the wire holding the cork of the bottle was removed, the cork was controlled with the index finger, the bottle was turned down first then turned up. Finally, the index finger was pulled while the bottle was at an angle of 45° with the vertical axis and the residue on the cork was discharged by the effect of pressure. The missing part was completed with its own wine. The sparkling wine was then corked and wired.

2.6. Determining the Number of Yeast

On the1st, 20^{th}, 40^{th}, 90^{th} and 180^{th} days of aging on yeast, 50 ml samples of champagne were taken aseptically in sterile containers and 1 mL of these samples were diluted up to 10^{-6} in 0.25% of saline. Then, the prepared dilution was spread on the malt extract agar by spreading method. This process was carried out in two parallel. Petri dishes were incubated at 25 ºC for 3-5 days. Colonies formed at the end of this period were determined (Campbell, 1988; Fleet, 1993).

Yeast count in alginate beads was made in accordance with the literature (Ciani and Ferraro, 1998). For this purpose, 2 beads were taken into test tubes and 1 mL of 2% EDTA and 1 ml of 3% NaCl solutions were added. The samples were mixed in a magnetic stirrer for 2 minutes. Then, 1mL was taken and diluted up to 10^{-6} in 0.25% salty water and planted on Malt Extract Agar. Petri boxes were left for 3-5 days of incubation at 25 ºC and the total yeast number was determined by counting the growing colonies.

In addition, the count of the yeasts to be added to the fermentation medium was made in accordance with the literature using methylene blue under microscope (Euromex, Nederland) with the help of Thoma slide (Ciani and Ferrora, 1998).

2.7. Free Amino Acid Analysis

Free amino acids (Aspartic acid, Glutamic acid, Asparagine, Serine, Glutamine, Histidine, Threonine, Arginine, Alanine, Tyrosine, γ-amino butyric acid, Ethanolamine, Valine, Methionine, Tryptophan, Phenylalanine, Isoleucine, Leucine, Lysine) were determined in accordance with the literature (Hermosin et al., 2003; Gomez-Alanso et al., 2007).

Derivatization process was applied to the wine samples first. 30 µL of diethyl ethoxy methylene malonate, 1.5 mL of methanol, 1 mL of wine sample, 3.5 mL of borate buffer (1M, pH 9) and 37.5 µL of α-amino butyric acid (internal standard) are placed in a 10 mL tube and the mouth of the tube capped and left in an ultrasonic bath at room temperature for 30 minutes. After this process, the sample was filtered through a 0.45 µm diameter filter and then injected into HPLC (Agilent 1100 model, USA) for analysis.

HPLC conditions: Operated at temperature 16 ºC with 5 µm particle size 250 mm length and 4.6 mm inner diameter ACE C18 column. Mobile phase was acetonitrile and acetate buffer (25 mM, pH = 5.8) while the flow rate was 0.9 mL / min and injected sample was amount of 50 µL.

Gradient program

Time (Minutes)	0.0	13.0	13.5	17.0	20.0	32.0
%A	6.0	16.0	18.0	18.0	22.0	32.0
%B	94.0	84.0	82.0	82.0	78.0	68.0

Acetate buffer (pH: 5.8): To prevent microorganism growth, 2.052 g of Na-Acetate and 0.2 g of Sodium Azide were dissolved in ultrapure water and the volume was made up to 1 L with ultrapure water. Subsequently, its pH was adjusted to 5.8 with 1 N HCl.

2.8. Isolation of Peptide Fractions

Wine samples were centrifuged along 10 minutes at 7000xg and 4 °C. Then, 45 mL of wine sample was passed through Amicon brand ultrafiltration (Millipore, Bedford, USA) device to separate fractions with a molecular weight of 10000 Da and peptide fractions were isolated. Free amino acid and peptide analyses were performed on the filtrate obtained. The filtrate was kept at -20 ºC until analysis.

2.9. Peptide Analysis

For peptide analysis, 1 mL was taken from the fractions below 10000 Da and taken part of fractions were subjected to acid hydrolysis with 25 µL internal standard (1.015 g/L (0.1 M HCl) and 2 mL 6 M HCl along 24 hours (Acedo et al., 1994; Perrot et al., 2002). Then, free amino acid analysis was performed in HPLC according to the method described above for the samples subjected to acid hydrolysis. The difference of free amino acid contents before and after acid hydrolysis was calculated and the peptide amount was determined.

2.10. Analysis of Data by Transforming Them into Images

Transforming data into images is the process of normalizing all data to 0-$2^{\text{bit depth}}$ or 0-1 range, and it is a method that has recently been used in the field of food engineering. Because it provides the opportunity to determine the statistically different classes visually. In addition, it provides advantages such as offering optimal value range instead of optimal value in the data set if used with image processing algorithms. Furthermore, if modelling is difficult due to fluctuations in the data set, the image interpolation algorithm helps to better understand the experimental results (Akben, 2018; Kalkan et. al, 2019).

In this study, the transforming data into images method and histogram equalization algorithm was used together to analyse the effects of free and immobilized yeasts on the contents of amino acids and amino acids in peptides in natural sparkling wines along the aging of base wine on yeasts. Transforming the data into a grayscale image can be done as in Equation 1. In the Equation 1, D is the data set consisting of n pieces of data like as $D = \{d_1, \quad d_2, \quad \cdots \quad d_n\}$

$$I(m) = 2^{bit\ depth-1} \times \frac{D}{max(D)} \qquad (1)$$

The maximum number of $I(m)$ in the Equation-1 corresponds to the white colour and the number zero corresponds to the black colour, while the numbers in between minimum and maximum numbers of $I(m)$ correspond to the shades (tones) of gray. Also, gray tones can be matched to colours from dark red to navy blue if desired. The visually distinguishable 8 main colours (Navy Blue, Blue, Cyan, Green, Yellow, Orange, Red, Dark Red) that are formed in case the grayscale image is coloured represent each class in the data set. In this way, the values in the data set can be classified visually (Akben, 2018; Kalkan et al., 2019).

On the other hand, histogram equalization is an algorithm used to equalize the gray tones that can be considered statistically the same as the colour tone representing the maximum value of the data set to a single colour tone. Thus, it enables easier tracking of value changes caused by an independent variable in the data set (Akben, 2018). Histogram equalization was used in this study to determine the day after which the amino acid contents reached its maximum.

3. Findings and Discussion

3.1. Growth of yeasts during the second fermentation

The growth of free and immobilized yeasts during the second fermentation of the base wine prepared using mixture of Emir + Dimrit grapes is given in Figure 1. As seen in Figure 1, S. *bayanus* and *S. oviformis* yeasts in alginate beads in the immobilized yeast experiment were measured as 6.12 log cfu/ml on day 1. Then, 7.03 log cfu/ml was measured for S. *bayanus* on day 20 and 6.74 log cfu/ml was measured for *S. oviformis* on same day (20th day). For free yeast application, S. *bayanus* and *S. oviformis* yeasts were measured as 6.29 and 6.20 log cfu/ml on day 1, respectively. Free yeast growth determined in the second fermentation is similar with results in the literature (Martinez-Rodriquez et al., 2002).

In their second fermentation trial using yeasts *Saccharomyces cerevisiae* EC 1118, J, P29, IFI 473 and IFI 475, the researchers stated that the number of live yeast was initially 6 log cfu/ml in all wines, but more than this value at 20th days. Then, on the 40th day, they reported that EC 1118 yeast could not be detected in the medium, but other yeasts continued to decrease and could not be detected in the medium on the 90th day. The results of the wine trials using with Emir + Dimrit (1: 1) grapes are consistent with these results in the literature. Only free *S. oviformis* yeasts showed similar numbers of yeast from day 1 to day 20. These examples differ from the literature. On the other hand, the yeasts passing from alginate beads to fermentation medium during the second fermentation of wine made with Emir + Dimrit mixture is given in Figure 2.

As seen in Figure 2, the number of S. *bayanus* and *S. oviformis* yeasts leaking from alginate beads into the fermentation medium were 2.36 and 3.07 log cfu/ ml on day 1, then 5.51 and 5.65 log cfu/ml on day 20. Thereafter, the number of yeasts started to decrease. If a general evaluation is made, it was determined that the immobilized S. *bayanus* and *S. oviformis* yeasts were denser in the fermentation medium than the free ones. In addition, the yeast numbers of the samples started to decrease after the 20th day, decreased significantly on the 40th day and the yeasts could not be isolated from the environment on the 90th day. This situation is similar in the literature (Martinez-Rodriguez et al., 2002; Bozdogan and Canbaş, 2011).

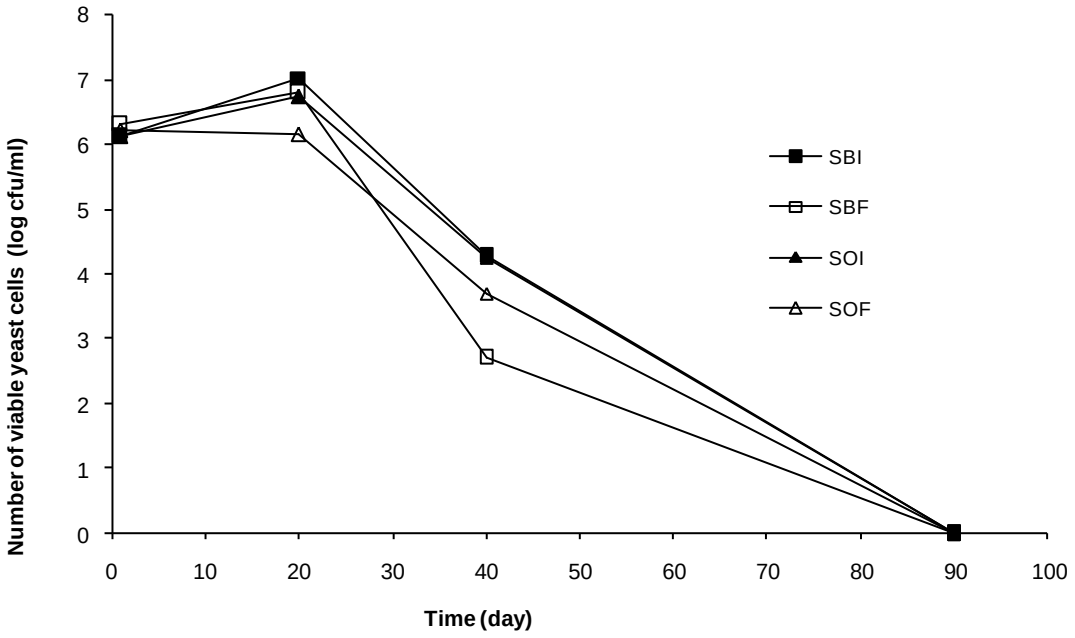

Figure 1. Growth of yeasts during second fermentation of base wine prepared using mixture of Emir + Dimrit grapes.

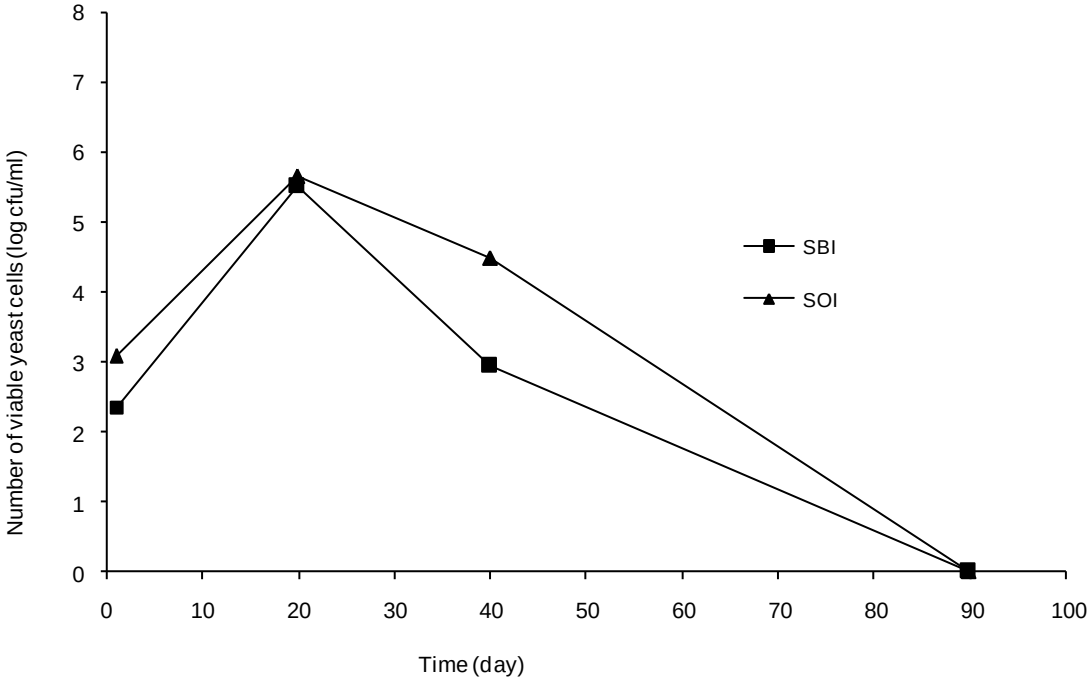

Figure 2. Yeasts passing from alginate beads to fermentation medium during the second fermentation of wine made with Emir + Dimrit mixture.

3.2. Changes in Amino Acid and Peptide Contents during the Second Fermentation

3.2.1. Analysis of Changes in Free Amino Acid Contents Depending on Yeast Type

First of all, the variation of each free amino acid depending on the yeast type and aging time was wanted to be analysed with polynomial surface models, but the determination coefficient (R^2 values) of the surface models were calculated between 0.089-0.84. Due to the insufficient determination coefficient, the measured amino acid values (raw data) were analysed by transforming them into images. Thus, the variation of each amino acid contents in wine depending on the yeast types and aging time was evaluated. Figure 3 and Figure 4 are for this purpose.

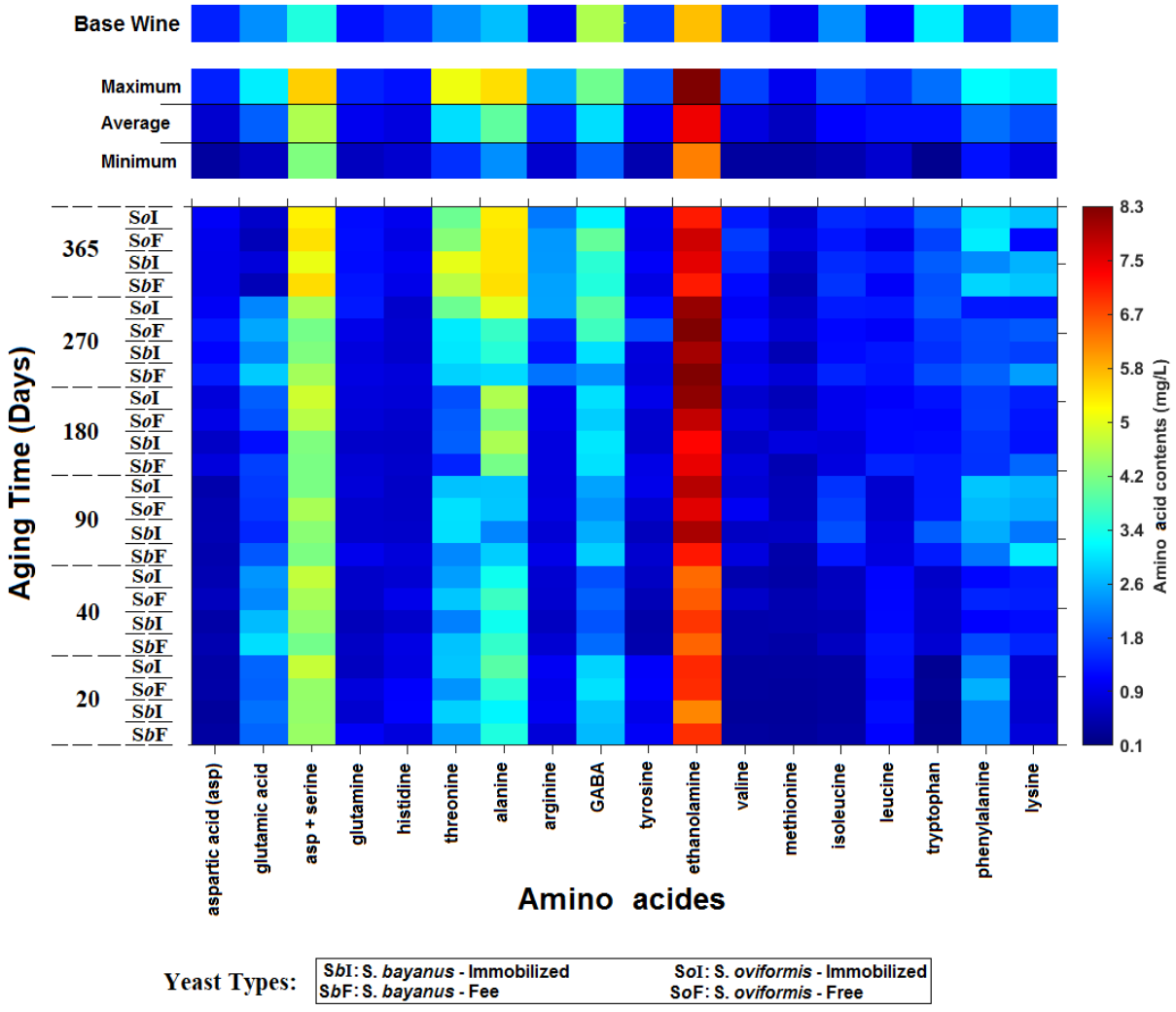

Figure 3. Image representation of the measured amino acid contents depending on aging time for all yeast types.

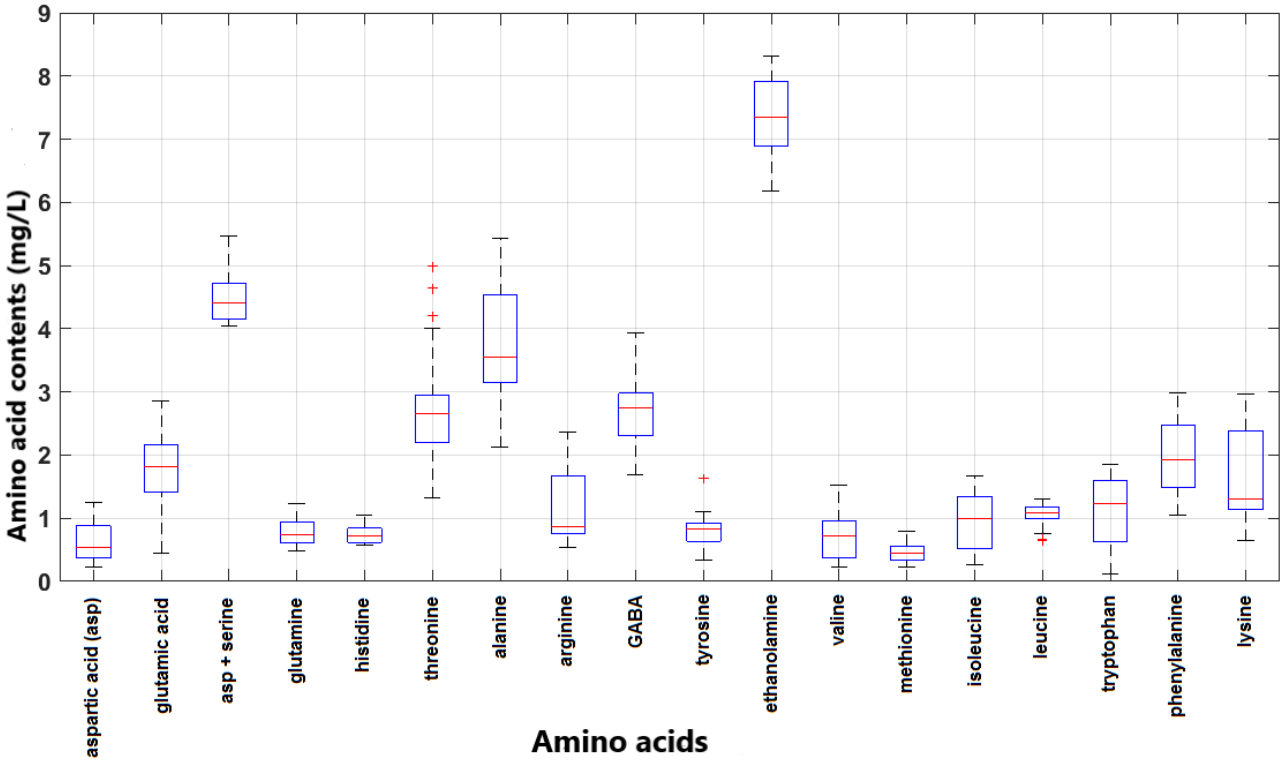

Figure 4. Box-plot graph showing the statistical coefficients of measured amino acid contents depending on day and for all yeast type

As can be seen in Figure 3, the amino acid with the highest content in wine is ethanolamine because ethanolamine values are represented in the image by the shade of orange-red colours corresponding to the change between 6.1-8.2 mg/L, and there are no other amino acids represented by these colours. In other words, ethanolamine content is the highest during the aging process for all yeast types, and this content is statistically different than other amino acid contents. Also, the contents of asp + serine, threonine, GABA and alanine wine are also statistically different from the other amino acids contents in wine as they are represented by the shade of green-yellow colours corresponding to the change between 1.2-5.5 mg/L. Therefore, they can be considered as a group with the second highest content in wine. Since the other amino acid contents are represented by blue and navy-blue shades corresponding to the change between 0.1-3 mg/L, it can be said that the contents of these amino acids in wine are in a single group consisting of two sub-groups that cannot be clearly separated from each other.

In summary, the contents of amino acids in wine during the aging time were classified into 3 main groups, which differ statistically significant from each other. In addition, if a group is formed for each color, it should be kept in mind that the contents of amino acids in wine can be divided into 8 groups that have a statistically significant difference from each other. However, distinguishing statistically these 8 groups from each other will be more difficult as compared to the 3 main groups that defined above. These analyses and related evaluations can also be seen from the statistical box-plots in Figure 4.

As shown in Figure 3, the colours representing the maximum content values obtained for asp, glutamine, histidine, GABA, tyrosine, valine, methionine, isolaine, laxine, triiopane are almost the same as those representing the amino acid contents of the base wine. This means that the content values measured at the end of the aging process for the specified amino acids are lower or not different than the content values of the amino acids in base wine. The colours representing the measured maximum content values for other amino acids are different from the representative colours for the base wine.

During the production of champagne, 4 yeast types (S. *bayanus*-Free, S. *bayanus*-Immobilized, S. *oviformis*-Free, S. oviformis-Immobilized) were tested. If so, the change in amino acid content for each of these 4 types can be modelled as a function of day then be analysed. Thus, a decision can be made about the changes in the number of amino acids caused by each yeast type, during the aging process. Therefore, the measured values of amino acids were averaged during the aging process and polynomial models were obtained for 4 yeast types. In Table 1 and Figure 5, polynomial models obtained for each yeast type and statistical coefficients of the models are shown.

Table 1. Aging Time dependent model equations of average amino acid contents for each yeast-cell combination

Yeast	**Cells**	**Fit**			
		$4.18 \times 10^{-6} \times t^2 - 0.0005382 \times t + 1.674$			
S. *bayanus*	Free	**R^2**	**Adj-R^2**	**SSE**	**RMSE**
		0.9612	0.9353	0.0172	0.0758
		$6.174 \times 10^{-6} \times t^2 - 0.0001097 \times t + 1.646$			
S. *bayanus*	Immobilized	**R^2**	**Adj-R^2**	**SSE**	**RMSE**
		0.9051	0.8419	0.0506	0.1299
		$2.539 \times 10^{-6} \times t^2 - 0.001025 \times t + 1.687$			
S. *oviformus*	Free	**R^2**	**Adj-R^2**	**SSE**	**RMSE**
		0.9416	0.9027	0.0229	0.0874
S. *oviformus*	Immobilized	$-6.467 \times 10^{-7} \times t^2 - 0.0002489 \times t + 1.608$			

R^2	Adj-R^2	SSE	RMSE
0.9154	0.8590	0.0436	0.1206

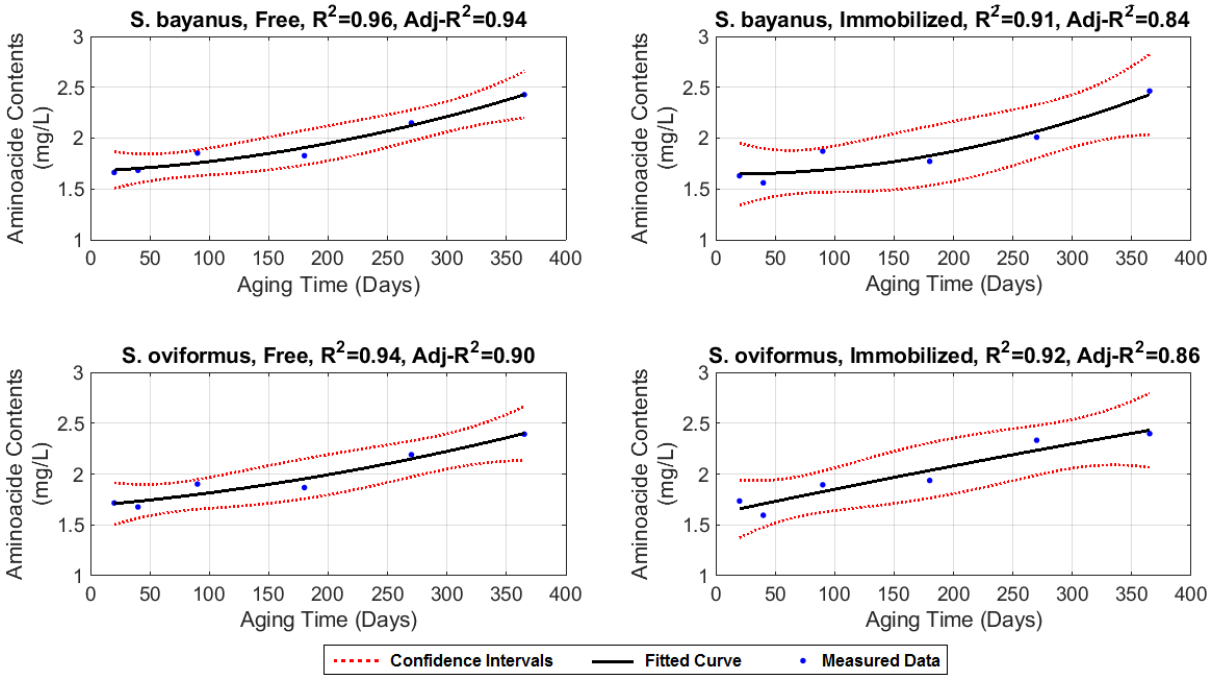

Figure 5. Polynomial models obtained for each yeast type

In all polynomial models in Figure 5, amino acid contents start from 1.65 and increase up to 2.43 mg / L at the end of the aging time. Since the average amino acid content in base wine is 2.115 mg / L, it is seen that more amino acids are obtained at the end of the aging time for all 4 yeast types compared to the base wine. In other words, the average amino acid content of the basic wine has increased at the end of the aging time for all 4 yeast types. According to the models, for the 4 yeast types, the aging days when the average amino acid content exceeds the amino acid content of the base wine are 269, 287, 258 and 218, respectively. However, Adj-R^2 values of the models in Figure 5 are between 0.84-0.94 and it cannot be said that the fit of the models is very good. Therefore, it would be more correct to make the analysis with image interpolation to make the determined values more accurate. Moreover, polynomial model equations present a single value for each critical aging day (It cannot collect the values that do not have statistically significant difference in a single group) and reduce the universality of the result. For these reasons, the measured values were first transformed into images in order to produce tolerant (universal) result, and these images were enlarged by bicubic interpolation. Thereafter, the days providing the highest amino acid content and the amount of amino acid content obtained in that days were determined by applying the histogram equalization algorithm to the images.

Figure 6 shows the representation of the average amino acid contents measured depending on the days for each yeast type with images enlarged by bicubic interpolation and the histogram equalization applied forms of these images. The enlarged image representations show the day-dependent change in amino acid content, and their histogram equalization applied forms determine the day intervals at which the maximum amino acid content is achieved.

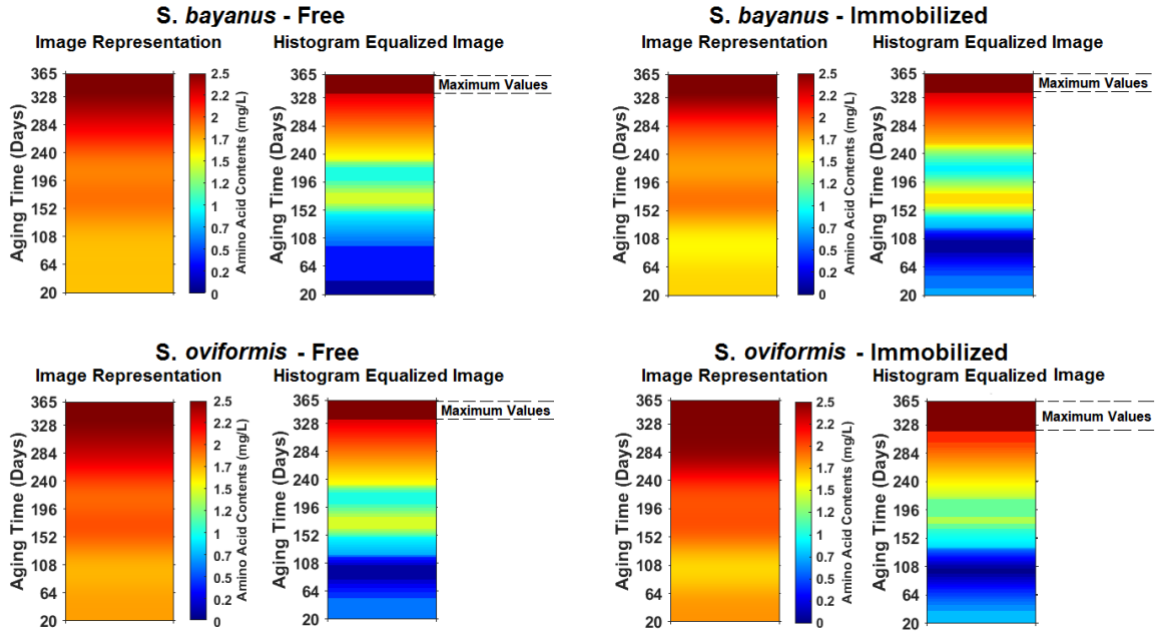

Figure 6. Representation of amino acid contents measured for each yeast type with magnified images and histogram equalization applied form of these images

As can be seen from Figure 6, while there are fluctuations (ups / downs) in amino acid content in the first 220 days of the aging period, only an increase is observed in other time intervals. The low coefficient of fit of the models in Figure 5 is also due to these fluctuations. Hence, the analysis with the image models in Figure 6 is more accurate.

The values represented in dark red in the histogram equalization applied images in Figure 6 are the highest values with no statistically significant difference between the amino acid contents. These values are in the range of 2.30-2.43 mg / L and were achieved for S. *bayanus*-Free, S. *bayanus*-Immobilized and S. *oviformis*-Free on days 335-365, while for S. *oviformis*-Immobilized on days 317-365. In other words, S. *oviformis*-Immobilized yeast provided the same amino acid content in a shorter time. However, on days that are indicated in dark red on the histogram equalized images and which statistically give almost the maximum number of amino acids, the amino acid contents obtained for all yeasts (2.30-2.43 mg/L) are higher than the amino acid content of the base wine (2.115 mg/L).

As a result, all the findings for amino acid content are given below for clarity.

- According to the analysis for each amino acid, the amino acid content was divided into 3 main groups in quantity. In these groups, ethanolamine with the highest amino acid content, the second highest asp + serine, alanine, teronine and GABA, and the third group are the others.
- The average amino acid content for all yeasts varied between 1.6-2.4 mg/L depending on the aging day.
- Although the average amino acid content varies according to the yeast type, amino acid contents exceeded the average amino acid content (2.115 mg/L) of the base wine on days 218-287 of aging time.
- The highest amino acid contents obtained using S. *bayanus*-Free, S. *bayanus*-Immobilized, S. *oviformis*-Free yeasts were achieved on days 335-365, while for S. *oviformis*-Immobilized yeast on day 317.

One of the striking findings is that the amino acid content fluctuates in the first 220 days of aging time. The reason for this is supported by studies in the literature. In these studies, the changes in nitrogenous compounds during aging the wines on yeast were examined and it was found that the amino acid contents decreased on the 20^{th} day. It has been reported that this situation can be explained by the yeast's use of amino acids in some metabolic events (Martinez-Rodriquez et. al., 2002). In another study about the occurrence of this fluctuation, it was emphasized that in the first 90 days of aging on yeast, the free amino acid contents decreased because yeasts use most amino acids for their reproduction (Martinez-Rodriquez and Polo, 2000; Feulliat and Charpentier, 1982; Leroy et. al., 1990; Herraiz and Ough, 1993). In another study, it was reported that during the second fermentation of the naturally sparkling wine obtained using Emir grapes, most of the free amino acids decreased between the 20^{th} and 40^{th} days of fermentation (Bozdogan and Canbaş, 2011). In another study examining the effect of wine aging process on amino acid and peptide contents, it was reported that the amino acid contents decreased after the 9th month of the aging time (Moreno-Arribas et al., 1998; Martinez-Rodriquez et al., 2002). As can be seen, literature studies are consistent with the fluctuations of the amino acid content in the first 220 days determined in this study.

Another finding obtained in the study is that the measured amino acid contents between 218-287 days of the aging period began to be higher than the amino acid content of the base wine. This finding is supported by another study in the literature. In the relevant literature study, it was determined that free amino acids pass into the wine medium in the first 6^{th} and 12^{th} months in aging period that lasts for 4 years (Martinez-Rodriquez and Polo, 2000). The consistency the results of literature studies and current study, show that the results of current study are also correct.

3.2.2. Analysis of Changes in Amino Acid Contents in Peptides Depending on Yeast Type

The analysis performed in the first step for free amino acids was repeated for the amino acids in the peptides in the second step of the study. The image representation of the amino acid contents measured in the peptides can be seen in Figure 7.

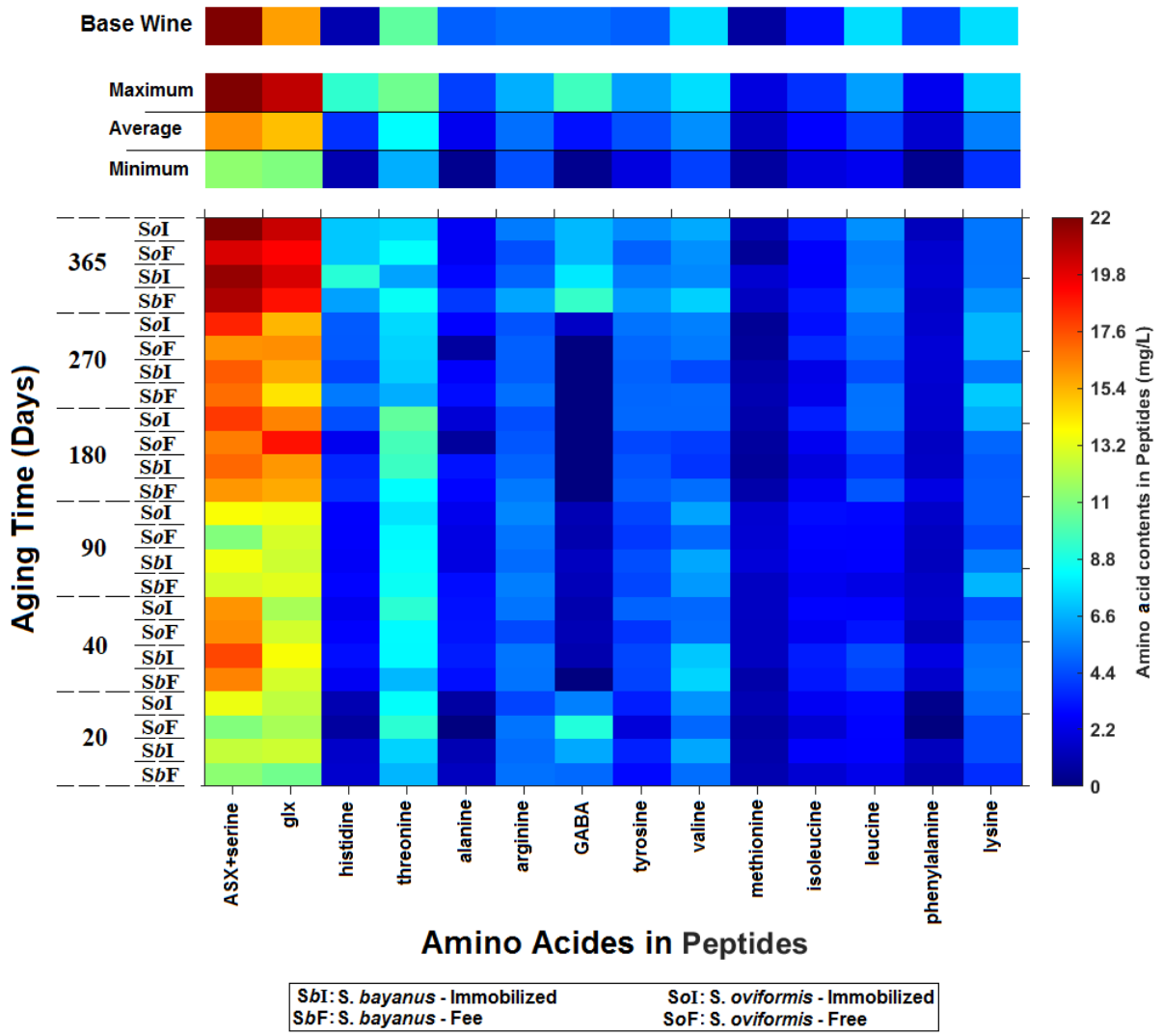

Figure 7. Image representation of the measured amino acid contents in peptides depending on aging time for all yeast types

As seen in Figure 7, the most abundant amino acids in terms of content are ASX + serine and glx, because the values of these amino acids are represented by the shadows of yellow-green-orange-red colours corresponding to the change between 1.5 and 22 mg/L and there are no other amino acids represented by these colours. In other words, the amino acids with the highest content in wine during the aging process are ASX + serine and glx and their content values are statistically significantly different from those of other amino acids. However, since the values of the teronine are represented by the shade of turquoise-green colours corresponding to the change between 6.3-10.4 mg/L, the

amount of content is statistically significantly higher than the remaining amino acids and can be considered as second group. Since the content amount of the remaining amino acids is represented by blue and dark blue tones corresponding to the change between 0-6.3 mg / L, the content amounts can be said to be in a single group.

In summary, the number of amino acids in the peptides were collected in 3 main groups with statistically significant differences from each other. These analyses can also be seen from the Box-plot chart in Figure 8.

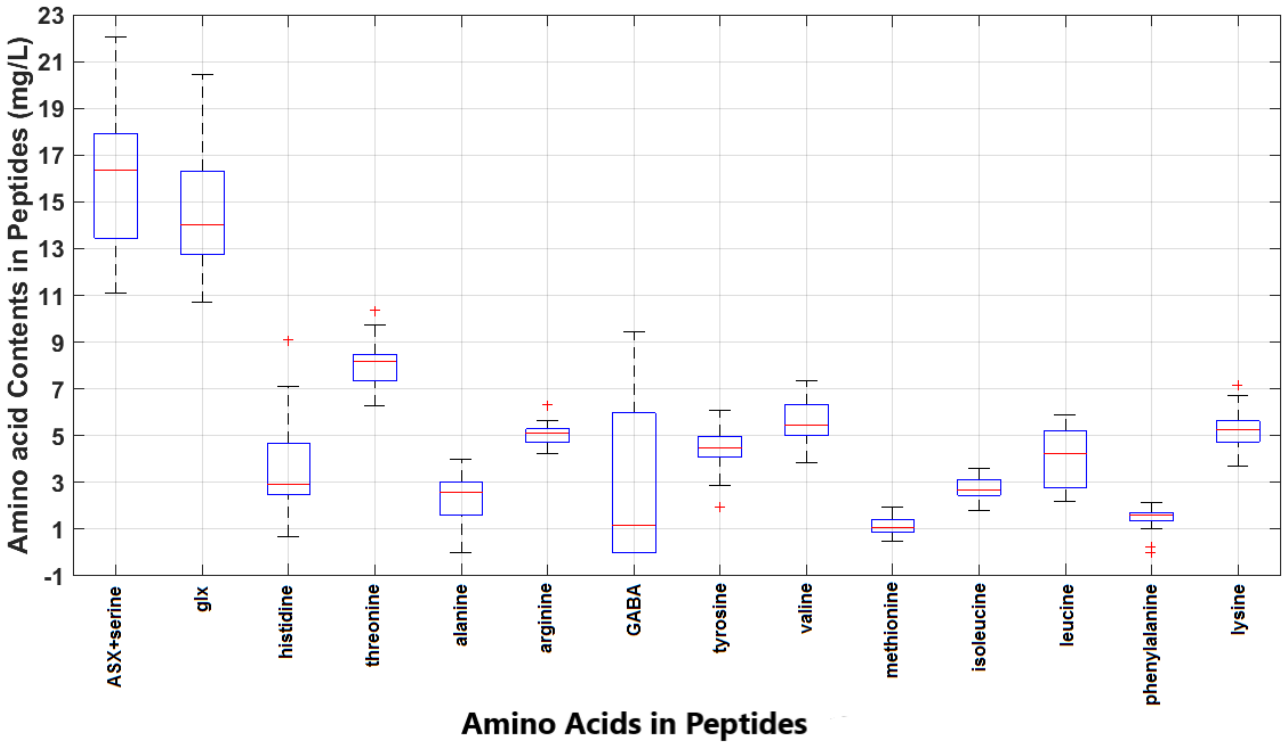

Figure 8. Box-plot graph showing the statistical coefficients of measured amino acid contents in peptides depending on day and yeast type

As shown in Figure 7, the colours representing the maximum content values obtained for asp, ASX + serine, teronine, alanine, valine, isoleucine, leucine, penylalanine and lysine are almost the same as those representing the amino acid contents of the base wine. The colours representing the maximum values measured for other amino acids are different from the colours representing the maximum values of amino acids in the base wine. In other words, while the aging process increased the number of amino acids glx, histidine, arginine, GABA, tyrosine and methylonine, it decreased or did not change the number of other amino acids.

In the study, it was tried to obtain polynomial models for each yeast type for analysis by using polynomial models as in Table 1 and Figure 5. The obtained polynomial models and the statistical coefficients of these models are shown in Table 2 and Figure 9.

Table 2. Aging Time dependent model equations of amino acid contents in peptides for each yeast-cell combination

Yeast	Cells	Fit			
		$2.214 \times 10^{-5} \times t^2 - 0.001204 \times t + 4.83$			
S. *bayanus*	Free	**R^2**	**Adj-R^2**	**SSE**	**RMSE**
		0.8599	0.7665	0.8263	0.5248
		$3.332 \times 10^{-5} \times t^2 - 0.00709 \times t + 5.502$			
S. *bayanus*	Immobilized	**R^2**	**Adj-R^2**	**SSE**	**RMSE**
		0.8094	0.6823	0.6658	0.4711
		$1.663 \times 10^{-5} \times t^2 - 0.0007618 \times t + 4.768$			
S. *oviformus*	Free	**R^2**	**Adj-R^2**	**SSE**	**RMSE**
		0.9277	0.8795	0.2344	0.2795
		$9.272 \times 10^{-6} \times t^2 + 0.002713 \times t + 4.86$			
S. *oviformus*	Immobilized	**R^2**	**Adj-R^2**	**SSE**	**RMSE**
		0.9280	0.8800	0.2822	0.3067

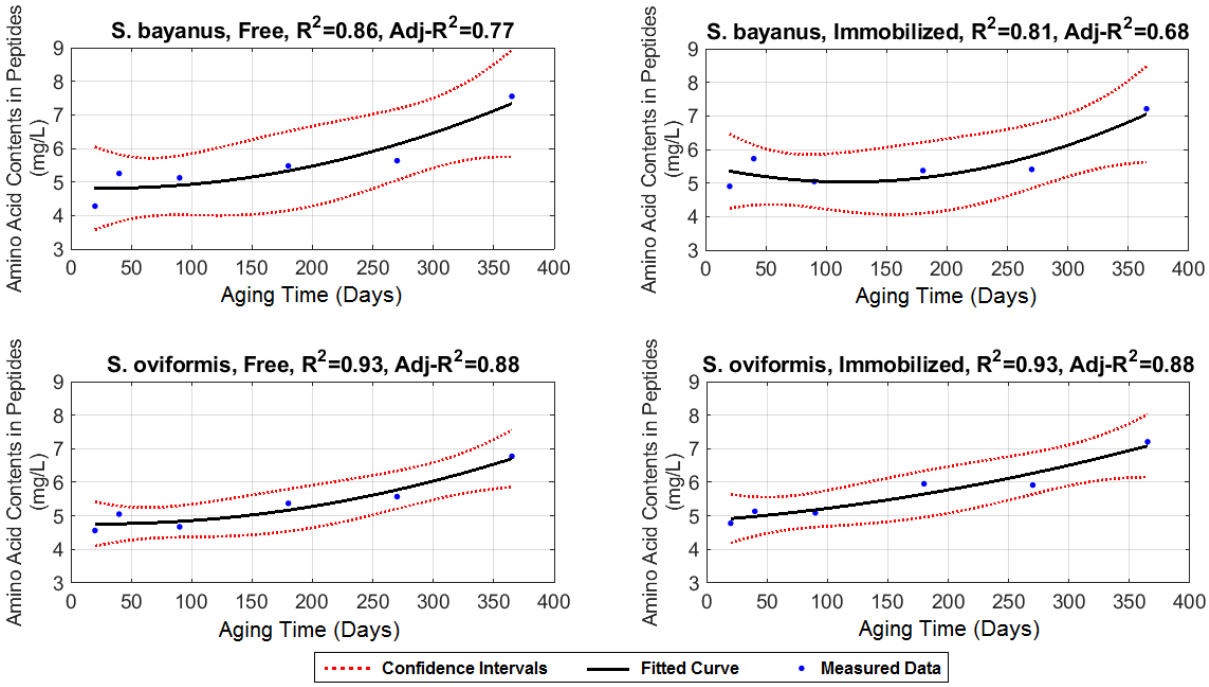

Figure 9. Polynomial models obtained for each yeast

In the polynomial models in Figure 9, the average amino acid contents in peptides changed between 4.28-7.55, 4.90-7.21, 4.56-6.77 and 4.77-7.20 mg / L for all yeasts, respectively but this is different from the Figure 3 because the change interval for each yeast is not the same. As compared to the average amino acid content (6.945 mg/L) in the base wine, it appears that slightly more amino

acids were obtained at the end of aging time for yeasts, except for S. *oviformis*-Free. According to the models, the days when the average amino acid contents exceed the average amino acid content in the base wine for S. *bayanus*-Free, S. *bayanus*-Immobilized, S. *oviformis*-Immobilized are 338, 359 and 351 days, respectively. However, Adj-R^2 values of the models in Figure 9 are between 0.68-0.88 and it cannot be said that the fit of the models is very good. Therefore, the measured values were first transformed into images and these images were enlarged by bicubic interpolation.

Then, histogram equalization was applied to the acquired images. Figure 10 shows the representation of the amino acid content measured for each yeast type with images enlarged by bicubic interpolation and the histogram equalization applied forms of these images.

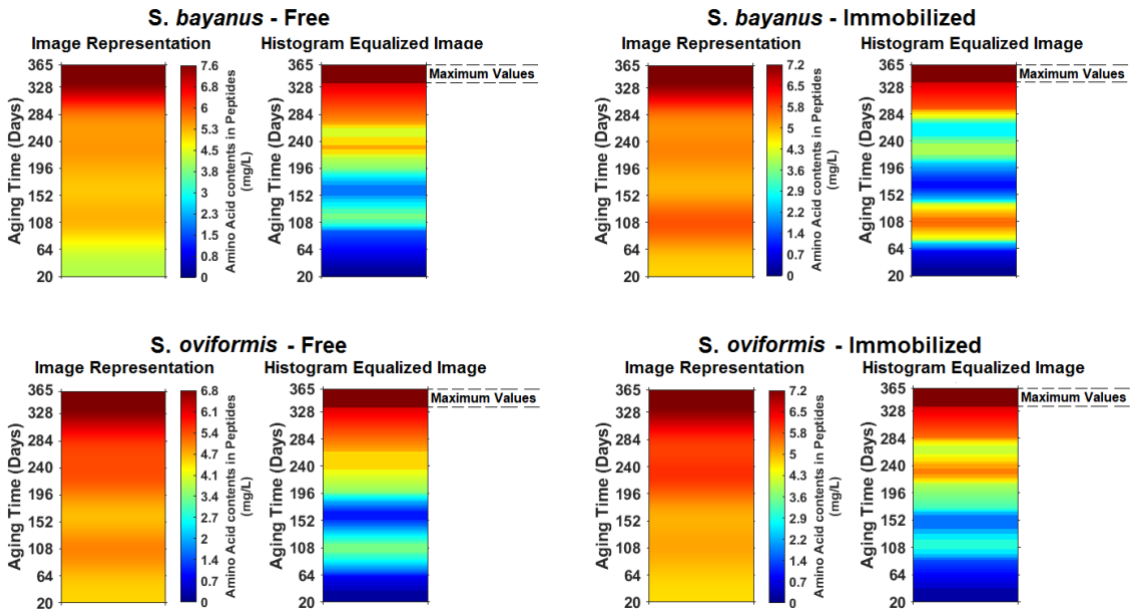

Figure 10. Representation of amino acid contents measured for each yeast with magnified images and histogram equalization applied form of these images

As can be seen from Figure 10, while there are fluctuations in the amino acid contents of peptides in the first 280 days of the maturation period, an increase is observed in other time intervals. This situation is similar to the change in Figure 6. The values represented with dark red in the histogram equalization applied images in Figure 10 are represent the values that do not have a statistically significant difference between the amino acid contents. These values occurred on days between 338 and 365 when the highest amino acid contents were achieved on average. In other words, the maximum amino acid contents were obtained from the 338th day for all yeasts. The highest amino acid contents determined in the specified day intervals are 6.95-7.55, 6.95-7.21, 6.3-6.77, 6.95-7.20 for *S. bayanus*-Free, *S. bayanus*-Immobilized, S. *oviformis*-Free, S. *oviformis*-Immobilized yeasts, respectively. Accordingly, the highest amino acid content was provided by *S. bayanus*-Free yeast,

and other yeasts, except for S. *oviformis*-Free yeast has provided more amino acid contents than the amino acid content (6.95 mg/L) of the base wine. As a result, all the findings for the amino acid contents in peptides are given below for clarity.

- As a result of the analysis for each amino acid, the content values were divided into 3 main groups. Among these groups, the highest amino acid content obtained is asx + serine and glx, the second is threonine and the third group is the others.
- Average amino acid contents obtained for the *S. bayanus*-Free, *S. bayanus*-Immobilized, S. oviformis-Free, S. oviformis-Immobilized yeasts were 7.55, 7.21, 6.77 and 7.20 mg/L, respectively, at the end of aging time.
- For *S. bayanus*-Free, *S. bayanus*-Immobilized, S. oviformis-Immobilized, the days when the average of amino acid contents exceed the average of amino acid contents of the base wine are days 338, 359 and 351, respectively. On the other hand, S. oviformis-Free caused to occur lower amino acid content than the average amino acid content (6.95 mg/L) of the basic wine at the end of the aging process.
- The highest average amino acid contents for all yeasts were achieved between days 338-365.
- Yeasts used more amino acids in peptides.

These findings about amino acids in peptides are also consistent with the findings in the literature. For example, in the literature, researchers have reported that there are fluctuations in number of peptides during the aging period, and this is due to the prolonged autolysis. In another study, it was determined that during the aging process of natural sparkling wine production made with Dimrit grape, amino acids in peptides decreased on the 20th day and then fluctuated until the 365th day (Bozdogan and Canbaş, 2012). Likewise, in another study, it was stated that the amino acid content in peptides fluctuated in the first 280 days. The finding obtained in the current study that yeasts benefit more from amino acids in peptides is also supported by studies in the literature. For example, in a study (Hidalgo et al., 2004) on the production of pink sparkling wine reported that yeasts used peptides more than amino acids during the second fermentation. The reason for this can be explained by a literature study that reports the breakdown and release of peptides in wines are due to the action of protease enzymes (Moreno-Arribas et al., 1996). Moreover, the release of peptides due to intracellular protease activity during fermentation also explains this situation (Alexandre et al., 2001).

In this study, it was determined that the contents of amino acids in the peptides reached the maximum between the 338-365 days. In a study in the literature, the change in the contents of peptides during the aging process of wines was examined and it was determined that the contents of peptides

reached the highest level in the 12th and 15th months of aging time (Bartolome et al., 1997). This result in the literature is similar to the result of the current study.

However, the finding of current study related to the effect of yeast strain and aging time on peptide content is striking. Similarly, in a study in the literature, it was determined that the effect of yeast strain and aging time on peptide content in the second fermentation was significant ($p < 0.05$) (Martinez-Rodriquez et al., 2002).

3.3. Foam sensory characteristics

The wine is considered to be of good quality if there is a lot of foam in the sparkling wine and it covers the entire surface of the wine. On the surface of this kind of bubbling wine, foam forms ring-shaped structure, gas bubbles are small, and foaming is fast(Gallart et al., 2004). Sensory evaluation results of sparkling wines are given in Table 3. The wines received acceptable sensory scores. In particular, sparkling wines produced with immobilized yeast appear to score higher. Hidalgo et al (2004) investigated the effects of different yeasts used in the second fermentation stage of sparkling wine production on the sensory properties of wines. The researchers stated that the sparkling wines obtained received acceptable scores in terms of sensory properties.

Table 3. Foam sensory characteristics of the sparkling wines at 12 months of aging time with yeast

Visual attributes	**SBF**	**SBI**	**SOF**	**SOI**
Inital Foam (1:Poor, 2:Normal, 3:Abundant)	$2.2^{c}\pm0.3$	$2.6^{b}\pm0.2$	$2.2^{c}\pm0.3$	$3.0^{a}\pm0.0$
Foam area (1:None,2:Partial,3:Total)	$2.0^{b}\pm0.0$	$2.6^{a}\pm0.2$	$2.2^{b}\pm0.3$	$2.7^{a}\pm0.3$
Foam collar(1:None,2:Partial,3:Total)	$1.9^{b}\pm0.5$	$2.5^{a}\pm0.4$	$1.9^{b}\pm0.5$	$3.0^{a}\pm0.0$
Bubble size(1:Large,2:medium,3:Small)	$2.2^{b}\pm0.4$	$2.7^{a}\pm0.4$	$2.0^{b}\pm0.0$	$2.7^{a}\pm0.4$
Effervescence speed(1:Slow,2:Medium,3:Slow)	$1.7^{b}\pm0.4$	$2.5^{a}\pm0.6$	$1.9^{b}\pm0.4$	$2.8^{a}\pm0.3$
Origin of bubbles(1:Glass wall,2:Glass wall and wine	$1.3^{c}\pm0.3$	$1.9^{ab}\pm0.2$	$1.5^{bc}\pm0.5$	$2.0^{a}\pm0.0$
Number of bubble chains (1:Lower than five, 2: Higher than five	$1.2^{b}\pm0.2$	$1.4^{ab}\pm0.2$	$1.2^{b}\pm0.3$	$1.7^{a}\pm0.4$
Global Impression(1:Bad,2:Acceptable,3:Good,4: Very good	$2.5^{c}\pm0.2$	$3.1^{b}\pm0.3$	$2.9^{bc}\pm0.5$	$3.7^{a}\pm0.3$

In the same row, the difference between the values indicated by different letters is statistically significant. ($p<0.05$).

4. Conclusions and Recommendations

In the study, natural sparkling wine was produced using a mixture (1:1) of Emir and Dimrit grapes, and the effects of free and immobilized yeast strains (*S. bayanus* and S. *oviformis*) that used in the second fermentation stage on free amino acid and peptide contents during the aging period were analysed. There is no significant difference between natural sparkling wines obtained using free and immobilized yeasts in terms of amino acids and amino acids in peptides. However, it should be noted that the process of removing yeast residue onto the cork that requires more labor is easier if immobilized yeast is used. On days between 335 and 365 of the aging period on yeast, the free amino acid content in natural sparkling wines was higher than that of the basic wine and reached its highest level. This result confirms how important the 1-year (365 days) aging period is mandatory for the production in the Champagne region. In addition, the results obtained also show that high quality natural sparkling wine can be produced using the mixture of Emir + Dimrit grapes. Sparkling wines with Emir+Dimrit blend (1:1) received acceptable sensory scores. It can be said that the foam quality of the immobilized yeasts used in the second fermentation stage is better than the free ones and that the Emir+Dimirt blend (1:1) suitable for the production of natural sparkling wine.

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Authors' Contributions

The contribution of the authors to the article should be indicated. (For example: All authors contributed equally to the study.)

Statement of Conflicts of Interest

No potential conflict of interest was reported by the authors.

Statement of Research and Publication Ethics

The author declares that this study complies with Research and Publication Ethics.

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