MITIGATION OF ACRYLAMIDE FORMATION IN BAKERY PRODUCTS BY USING ASPARAGINASE ENZYME

ASPARAJİNAZ ENZİMİ KULLANILARAK FIRINCILIK ÜRÜNLERİNDE AKRİLAMİD OLUŞUMUNUN AZALTILMASI

SELAHATTİN GAZİ

DR NESLİHAN TAŞ

Supervisor

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ABSTRACT

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Selahattin GAZİ

Master of Science, Department of Food Engineering

Supervisor: Dr. Neslihan TAŞ

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Maillard reaction is a reaction that occurs as a result of the interaction of amino groups and carbonyl groups. It improves the characteristics of foods such as color, taste, smell and texture although it causes some undesirable effects such as losses in the nutritional value of food and the formation of thermal process contaminants including acrylamide. Acrylamide is a carcinogenic, genotoxic, and cytotoxic compound formed by the reaction of asparagine and carbonyl compounds such as reducing sugars.

The presence of asparagine, the precursor of acrylamide, in bakery products in high amounts leads to the formation of high amounts of acrylamide during the baking process. The high consumption of bakery products in the diet makes the development of strategies to reduce acrylamide in bakery products essential. The expectation of enactment of legal regulations regarding the limitation of the amount of acrylamide in bakery products in the

future reveals acrylamide as a problem that needs to be solved for the industry. Asparaginase enzyme, which is envisaged as a tool for acrylamide reduction strategy, stands out as a good option because it does not require any changes in heat treatment parameters and product formulations, preserves sensory properties, and does not have to be specified on the product label.

Within the scope of this thesis, applications were made by using commercial asparaginase enzyme in high water content hard biscuits, low water content hard biscuits, soft biscuits, pretzel-like snacks, and wafers to reduce asparagine in the dough of these products and accordingly to restrict acrylamide formation. For this purpose, changes were made on enzyme dosage, enzyme application time, resting time and temperature, dough mixing speed and time, and in the order of addition of the recipe components during dough preparation.

As a result of these applications, acrylamide formation was reduced by 96% in the high water content hard biscuit, in which 3000 ASNU/kg flour asparaginase dose was applied and baked after 15 min resting time. In soft biscuits, 54% reduction in acrylamide was achieved with the application of 5000 ASNU/kg flour asparaginase dose and changing mixing order. Asparaginase dose of 3000 ASNU/kg flour and then 15 min resting time applied to the pretzel-like snack limited the formation of acrylamide by 80% after baking. A significant reduction of acrylamide could not be achieved in low water hard biscuits.

The correlation coefficients between asparagine and acrylamide in high water content hard biscuit, low water content hard biscuit, soft biscuit, and pretzel-like snack were determined as 0.74, 0.03, 0.64, and 0.91, respectively. It was revealed that there was a strong positive interrelation between asparagine and acrylamide content in all products except for low water content hard biscuits where the asparaginase was not active. In the wafer, only asparagine analysis was performed and with 1000 ASNU/kg flour enzyme treatment 97% asparagine reduction was achieved. It was determined that the potential acrylamide formation reduction would be high.

When the effects of water activity and pH on the activity of asparaginase in the dough of bakery products were examined, water activity was found to be more effective than pH.

Additionally, application of asparaginase was found to have no adverse effect on the product quality parameters such as surface color and spread ratio.

As a result of this study, high rates of acrylamide reduction were achieved in bakery products by using the asparaginase enzyme. This strategy could be used as a guide for the food industry to reduce acrylamide in bakery products.

Keywords: Maillard reaction, acrylamide, asparagine, asparaginase, bakery products

ÖZET

ASPARAJİNAZ ENZİMİ KULLANILARAK FIRINCILIK ÜRÜNLERİNDE AKRİLAMİD OLUŞUMUNUN AZALTILMASI

Selahattin GAZİ

Yüksek Lisans, Gıda Mühendisliği Bölümü

Tez Danışmanı: Dr. Neslihan TAŞ

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Maillard reaksiyonu, amino grupları ile karbonil gruplarının etkileşimi sonucu meydana gelen bir reaksiyondur. Gıdaların renk, tat, koku ve tekstür gibi karakteristik özelliklerini iyileştirmesine rağmen gıdaların besin değerinde kayıplara ve akrilamid gibi ısıl işlem kontaminantlarının oluşumu gibi istenmeyen etkilere neden olmaktadır. Akrilamid, asparajin ve indirgen şekerler gibi karbonil bileşiklerinin reaksiyonu ile oluşan kanserojen, genotoksik ve sitotoksik bir bileşiktir.

Fırıncılık ürünlerinde akrilamidin öncülü olan asparajinin yüksek miktarda bulunması, pişirme işlemi sırasında yüksek miktarda akrilamid oluşumuna yol açmaktadır. Diyette fırıncılık ürünlerinin yüksek miktarda tüketimi, fırıncılık ürünlerinde akrilamidin azaltılmasına yönelik stratejilerin geliştirilmesini zorunlu kılmaktadır. Gelecekte fırıncılık ürünlerindeki akrilamid miktarının sınırlandırılmasına ilişkin yasal

düzenlemelerin yapılması beklentisi, akrilamidin endüstri için çözülmesi gereken bir sorun olduğunu ortaya koymaktadır. Akrilamid azaltma stratejisi için bir araç olarak öngörülen asparajinaz enzimi, ısıl işlem parametrelerinde ve ürün formülasyonlarında herhangi bir değişiklik yapmayı gerektirmemesi, duyusal özellikleri koruması ve ürün etiketinde belirtilmesinin gerekmemesi gibi nedenlerle iyi bir seçenek olarak öne çıkmaktadır.

Bu tez kapsamında yüksek su oranlı sert bisküviler, düşük su oranlı sert bisküviler, yumuşak bisküviler, pretzel benzeri atıştırmalıklar ve gofretlerde ticari asparajinaz enzimi kullanılarak bu ürünlerin hamurlarındaki asparajinin azaltılması ve buna bağlı olarak akrilamid oluşumunun kısıtlanması için uygulamalar yapılmıştır. Bu amaçla, hamur hazırlama sırasında enzim dozajı, enzim uygulama süresi, dinlenme süresi ve sıcaklığı, hamur karıştırma hızı ve süresi ile reçete bileşenlerinin eklenme sırası üzerinde değişiklikler yapılmıştır.

Yapılan işlemler sonucunda 3000 ASNU/kg un asparajinaz dozu uygulanan ve 15 dakika dinlendirilerek fırınlanan yüksek su oranlı sert bisküvide akrilamid oluşumu %96 oranında azaltılmıştır. Yumuşak bisküvide 5000 ASNU/kg un asparajinaz dozu ve karıştırma sırasının değiştirilmesi uygulamasıyla akrilamid %54 oranında azaltılmıştır. 3000 ASNU/kg un asparajinaz dozu ve ardından 15 dakika dinlendirme uygulanan pretzel benzeri atıştırmalıklarda fırınlanma sonucunda akrilamid oluşumu %80 oranında sınırlandırılmıştır. Düşük su oranlı sert bisküvide akrilamidde anlamlı bir akrilamid azalma sağlanamamıştır.

Yüksek su oranlı sert bisküvi, düşük su oranlı sert bisküvi, yumuşak bisküvi ve pretzel benzeri atıştırmalıkta asparajin ve akrilamid arasındaki korelasyon katsayıları sırasıyla 0.74, 0.03, 0.64 ve 0.91 olarak saptanmıştır. Asparajinazın aktif olmadığı düşük su oranlı sert bisküviler hariç tüm ürünlerde asparajin ve akrilamid içeriği arasında güçlü bir pozitif ilişki olduğu ortaya çıkmıştır. Gofrette ise yalnızca asparajin analizi yapılmış ve 1000 ASNU/kg un enzim uygulaması ile asparajinde %97 oranında azalma sağlanmıştır. Buna göre gerçekleşebilecek potansiyel akrilamid oluşumundaki azalmanın yüksek olacağı belirlenmiştir.

Fırıncılık ürünlerinin hamurlarındaki asparajinaz aktivitesi üzerine su aktivitesi ve pH'nın etkileri incelendiğinde, su aktivitesinin pH'dan daha etkili olduğu saptanmıştır. Ayrıca asparajinaz uygulamasının yüzey rengi ve yayılma oranı gibi ürün kalite parametreleri üzerinde olumsuz bir etkisinin olmadığı bulunmuştur.

Bu çalışma sonucunda, asparajinaz enzimi kullanılarak fırıncılık ürünlerinde yüksek oranlarda akrilamid indirgenmesi sağlanmıştır. Bu strateji, gıda endüstrisine fırıncılık ürünlerinde akrilamidin azaltması için bir kılavuz olarak kullanılabilir.

Anahtar Kelimeler: Maillard reaksiyonu, akrilamid, asparajin, asparajinaz, fırıncılık ürünleri.

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CONTENT

ABSTRACT i

ÖZET iv

ACKNOWLEDGEMENTS vii

CONTENT viii

LIST OF FIGURES xi

LIST OF TABLES xii

SYMBOLS AND ABBREVIATIONS xiv

1. INTRODUCTION 1

2. GENERAL INFORMATION 2

2.1. Maillard Reaction 2

2.1.1. Mechanism of Maillard Reaction 3

2.1.2. Factors Affecting Maillard Reaction 5

2.2. Acrylamide 7

2.2.1. Acrylamide Formation Mechanism 8

2.2.1.2. Major Pathways of Acrylamide Formation 8

2.2.1.3. Minor Pathways of Acrylamide Formation 10

2.2.2. Factors Effecting the Formation of Acrylamide 11

2.2.2.1. Reducing Sugars 11

2.2.2.2. Amino Acids 11

2.2.2.3. Lipids 12

2.2.2.4. Moisture Content and Water Activity 12

2.2.2.5. pH 12

2.2.2.6. Thermal Load 12

2.3. Acrylamide in Bakery Products 12

2.3.1. Mitigation Strategies of Acrylamide in Bakery Products 14

2.3.1.1. Agronomy 15

2.3.1.2. Processing 16
2.3.1.3. Recipe 16
2.4. Acrylamide Mitigation By L-Asparaginase 18
3. MATERIALS AND METHODS 21
3.1. Chemical and Consumables 21
3.2. Preparation of the Dough and Baking 21
3.3. Measurement of Spread Ratio 25
3.4. Analysis of Color 25
3.5. pH Measurement 25
3.6. Analysis of Water Activity 26
3.7. Analysis of Acrylamide 26
3.8. Analysis of Asparagine 27
3.9. Statistical Analysis 28
4. RESULTS AND DISCUSSION 29
4.1. Effect of Asparaginase Treatments on Acrylamide Reduction in Bakery Products and Asparagine Reduction in Their Dough 29
4.1.1. Effect of Asparaginase Treatments on Acrylamide Reduction in Sheeted Dough Biscuits and Asparagine Reduction in Sheeted Dough 29
4.1.2. Effect of Asparaginase Treatments on Acrylamide Reduction in Rotary Dough Biscuits and Asparagine Reduction in Rotary Dough 34
4.1.3. Effect of Asparaginase Treatments on Acrylamide Reduction in Wire-Cut Biscuits and Asparagine Reduction in Wire-Cut Dough 37
4.1.4. Effect of Asparaginase Treatments on Acrylamide Reduction in Pretzel-like Snack and Asparagine Reduction in Pretzel-like Snack Dough 41
4.1.5. Effect of Asparaginase Treatments on Asparagine Reduction in Wafer Dough 43
4.2. Effect of Water Activity on Asparagine Content and Acrylamide Reduction in Bakery Products 44
4.3. Effect of pH on Asparagine Content and Acrylamide Reduction in Bakery Products 46
4.4. Interrelation Between Asparagine Content of Dough and Acrylamide Content of Bakery Products 50

4.5. Effect of Acrylamide Reduction Strategies on Quality Parameters of Bakery Products 52
4.5.1 Spread Ratio 52
4.5.2 Color 56
5. CONCLUSION 61
6. REFERENCES 64
ANNEX 75
Pubblications 75
CURRICULUM VITAE 76

LIST OF FIGURES

Figure 2.1. Diagram of MR mechanism, adopted from [8]. 3
Figure 2.2. Acrylamide formation mechanism via Maillard reaction, adopted from [40]. 9
Figure 2.3. Minor pathways of acrylamide formation, adopted from [47]. 10
Figure 2.4. Acrylamide mitigation methods in bakery product. 15
Figure 2.9. Mechanism of the L-asparaginase, adopted from [74]. 19
Figure 4.1. Interrelation between asparagine content of the sheeted dough (mg/kg) and acrylamide content of sheeted dough biscuits (µg/kg). 50
Figure 4.2 Interrelation between asparagine content of the rotary dough (mg/kg) and acrylamide content of rotary dough biscuits (µg/kg). 51
Figure 4.3 Interrelation between asparagine content of the wire-cut dough (mg/kg) and acrylamide content of wire-cut biscuits (µg/kg). 51
Figure 4.4 Interrelation between asparagine content of the pretzel-like snack dough (mg/kg) and acrylamide content of pretzel-like snack (µg/kg). 52

LIST OF TABLES

Table 4.1. Acrylamide content (µg/kg sample) and acrylamide reduction (%) in sheeted dough biscuits after asparaginase enzyme treatments. 29
Table 4.2. Asparagine concentration (mg/kg dough) and asparagine reduction (%) in sheeted dough biscuits after asparaginase enzyme treatments. 32
Table 4.3. Acrylamide content (µg/kg sample) and acrylamide reduction (%) in rotary dough biscuit after asparaginase enzyme treatments. 34
Table 4.4. Asparagine concentration (mg/kg dough) and asparagine reduction (%) in rotary dough biscuit after asparaginase enzyme treatments. 35
Table 4.5. Acrylamide content (µg/kg sample) and acrylamide reduction (%) in wire-cut biscuit after asparaginase enzyme treatments. 37
Table 4.6. Asparagine concentration (mg/kg dough) and asparagine reduction (%) in wire-cut biscuit after asparaginase enzyme treatments 39
Table 4.7 Acrylamide content (µg/kg sample) and acrylamide reduction (%) pretzel-like snack after asparaginase enzyme treatments. 41
Table 4.8 Asparagine concentration (mg/kg dough) and asparagine reduction (%) in pretzel-like snack after asparaginase enzyme treatments. 42
Table 4.9. Asparagine concentration (mg/kg dough) and asparagine reduction (%) in wafer after asparaginase enzyme treatments. 43
Table 4.10. Water activity of different dough types. 45
Table 4.11. pH values of sheeted dough samples. 46
Table 4.12. pH values of rotary dough samples. 47
Table 4.13. pH of wire-cut dough samples. 48
Table 4.14. pH of pretzel-like snack dough samples. 48
Table 4.15. pH of wafer dough samples. 49
Table 4.16. Spread ratio of sheeted dough biscuits. 53
Table 4.17. Spread ratio of rotary dough biscuits. 53
Table 4.18. Spread ratio of wire-cut biscuits. 54
Table 4.19. Spread ratio of pretzel-like snack. 55
Table 4.20. L, a, b color values of sheeted dough biscuit samples. 56

Table 4.21. L, a, b color values of rotary dough biscuit samples..... 57
Table 4.22. L, a, b color values of wire-cut biscuit samples..... 58
Table 4.23. L, a, b color values of pretzel-like snack samples. 59
Table 4.24. Control and most effective enzyme treatment photograph of bakery products. 60

SYMBOLS AND ABBREVIATIONS

Symbols

μ	Micro

Abbreviations

NBR	Non-enzymatic Browning Reactions
MR	Maillard Reaction
ARP	Amadori Rearrangement Product
SD	Strecker Degradation
MRIP	Maillard Reaction Intermediate Products
Min	Minute
FAO	Food and Agriculture Organization
WHO	World Health Organization
IARC	International Agency for Research on Cancer
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
GRAS	Generally Recognized as Safe
ASNU	Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions
ASPU	Amount of L-asparaginase that free one micromole of ammonia from asparagine per minute under standard conditions)
S	Shortening
RT	Resting Time
MS	Mixing Speed
MT	Mixing Time
MOC	Mixing of Order Change

1. INTRODUCTION

Maillard reaction between amino acids and reducing sugars provides some desirable characteristics such as color, taste, and odor to foods. However, it is also responsible from the formation of carcinogenic compounds simultaneously. Acrylamide is one of the compounds that is harmful to health forms a result of the Miallard reaction. Therefore, reducing the formation of acrylamide in foods is of importance. Due to the presence of asparagine, which is the acrylamide precursor, in higher levels in bakery products, acrylamide formation is inevitable in bakery products which are subjected to heat treatments.

Within the scope of this thesis, applications related to the reduction of acrylamide formation by converting asparagine to aspartic acid by using asparaginase enzyme were perfomed. For this purpose, enzymatic processes were applied to high water content hard biscuits, low water content hard biscuits, soft biscuits, pretzel-like snacks, and wafers. In these applications, changes were made in enzyme dosage, enzyme application time, resting time and temperature, dough mixing speed and time, and the mixing order of ingredients to increase enzyme activity. In these applications, the interrelation between asparagine and acrylamide was revealed. Water activity and pH measurements were made to observe the effect of water activity and pH on the activity of asparaginase in enzymatic processes. Color and physical properties of bakery products whose dough was treated with asparaginase enzyme were determined and it was questioned whether asparaginase treatments had a negative effect on quality or not.

2. GENERAL INFORMATION

2.1. Maillard Reaction

Since ancient times, foods have been subjected to heat treatments with the aim of extending the shelf life, removing pathogens, enzymes, increasing digestibility, improving taste, odor, appearance, and texture [1]. During the heat processing of foods, non-enzymatic browning reactions (NBR) occur, and these browning reactions transform the appearance, flavor, and nutritional value of the food in a positive or negative way [2]. Maillard reaction (MR) is an important NBR which occurs between amino groups and carbonyl groups in food [2,3].

MR was discovered by chance in 1912 by L. C. Maillard, who investigated optimal chemical conditions for peptide synthesis. Maillard observed the reaction of various chemical compounds with amino acids, and then assumed that when sugars and amino acids were brought together, peptides would be synthesized. However, when he heated this sugar-amino acid mixture above 170 ºC, he found that a rapid browning occurred, and carbon dioxide escaped from the mixture. After then, he concluded that this reaction was a novel reaction unrelated to peptide production. Nonetheless, Maillard could not specifically identify the browning compounds formed as a result of the reaction he discovered [4].

In the following years, number of publications associated with MR increased. Unraveling the mechanism of MR, investigating the effects of the resulting products on human health, improving the quality and sensory properties of foods, understanding the changes in nutritional value of food, and maximizing or minimizing the MR in foods according to the desired benefits are among the reasons for this increase [3]. Furthermore, the findings from these studies revealed that the MR cannot occur only between amino acids, peptides, protein side chains and reducing sugars, aldehydes, ketones in foods, but also between amino and carbonyl compounds in living organisms. This non-enzymatic browning reaction, which occurs in living systems, is called as glycoslylation. Glycoslylation causes body proteins to lose their function and gives rise many disorders due to the accumulation of advanced glycation end products [2,5].

MR helps to create the desired taste, odor, and color in foods and thus increases the attractiveness of foods for consumers. However, although such advantages, the presence of disadvantages such as formation of toxic compounds (acrylamide, 5-hydroxymethylfurfural (HMF)) and losses in nutritional value indicates that the MR should be kept under control. Knowledge on how MR occurs in detail is essential to balance these two opposite situations [6,7].

2.1.1. Mechanism of Maillard Reaction

The first comprehensive study explaining the MR mechanism was performed by Hodge [8], which remains valid today. MR has a very complex mechanism, but Hodge [8] classified the reaction into 3 consecutive basic steps. Still today, Hodge diagram is the most widely used diagram in the visual representation of the mechanism of MR (Figure 2.1).

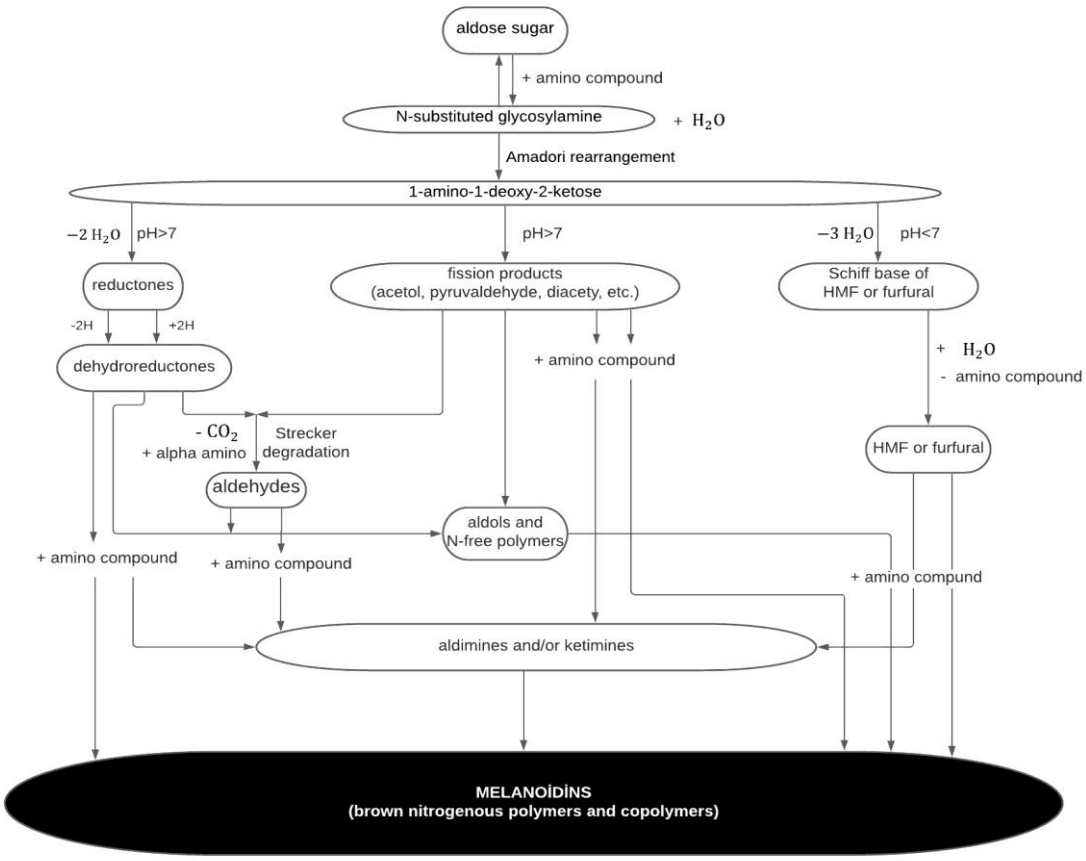


Figure 2.1. Diagram of MR mechanism, adopted from [8].

The first step of the reaction, called the initial stage, begins with the interaction of amino groups of amino acids and carbonyl groups of reducing sugars to create amine-sugar condensation, and the result of this condensation forms Schiff base which cyclized to N-substituted glycosylamine [8]. Then, if the source of the carbonyl group is an aldose sugar, Amodori product (1-amino-1-deoxy-ketose) is formed after amine-sugar condensation. If the source of the carbonyl group is a ketose sugar, Heyns product (2-amino-2-deoxy-aldose) is formed after amine-sugar condensation [9].

Amadori products are one of the first stable products form during MR and are used as a marker for monitoring nutritional value and quality loss of some foods [10]. For instance, the Amadori product, which is formed as a result of condensation of lysine and reducing sugars, is called as N- Ɛ-fructoselylysine (which is measured as furosine) and is used in the study of quality losses of cookies [11].

During the progression phase, the second step of the MR, Amadori rearrangement product (ARP) follows different paths with changing of pH. In acidic pH, ARP tends to follow 1,2-enolization pathway. As a result of 1,2-enolization, 3 molecules of water are removed from the ARP structure and highly reactive compounds such as 3,4-dideoxyhexosulose are formed. 3,4-Dideoxyhexosulose is not stable and turns into various compounds such as HMF and furfural [8,12]. In alkaline pH, ARP tends to follow 2,3-enolization pathway. As a result of 2,3-enolization, reductones, dehydroreductones, and fission products (diacetyl, pyruvaldehyde, and acetol) are formed. These compounds interact with free amino groups and amino ketones are formed [8,12].

In addition, highly reactive α-dicarbonyl compounds such as 3-deoxyglucosone and 1-deoxyglucosone are formed after retro-aldol condensation following these two enolizations [13]. α-Dicarbonyl compounds are not only produced in the intermediate step of the MR but can also be produced from sugar caramelization reactions when there is no amino acid in the medium. α-Dicarbonyl compounds are also formed as a result of lipid oxidation. α-Dicarbonyl compounds can participate in Strecker degradation even they arise from these two different production pathways [14,15].

Another reaction that occurs in the intermediate step of MR is the Strecker degradation (SD). SD is the reaction between α-dicarbonyl compounds, which are formed consequence of degradation of ARP, and α-amino acids. During Strecker degradation, amino acids oxidize and compounds such as aldehydes, which contribute significantly to flavor and aroma, are formed. Additionally, carbon dioxide forms through decarboxylation of amino acids during Strecker degradation [8,16].

In the third and last step of the MR, aldehydes, aldimines, ketoimines and other unstable and reactive compounds formed in the intermediate steps proceeds to further reactions such as aldol condensation, aldehyde-amine condensation, cyclization, polymerization. As a result, high molecular weight dark nitrogenous polymer, namely melanoidin, is formed. The chemical structure of the melanoidin is quite complex because it is a polymer structure with many MR product units. Studies on melanoidins suggests that melanoidins have positive impacts on human health due to its high antioxidant capacity, oxidative stress reduction, and chelation with metals [7,17].

2.1.2. Factors Affecting Maillard Reaction

There are many parameters that cause inhibition or progress of MR. The pH of the matrix where MR takes place is important factor that identifies the direction of MR. Alkaline conditions, in which amino groups of amino acids are in the simplest form and in which reducing sugars lose their cyclic form and switch to open chain form, promote MR to fission products and reductons [2]. With the decrease of pH, the formation of furfural in the MR suppresses the formation of reductones, thus affects the resulting Maillard reaction products (MRP) [18]. The elevation of ambient pH accelerates MR and increases browning intensity. In the study conducted by Ajandouz and Puigserver [19], it was shown that the amount of amino acid and reducing sugar decreased more and a darker end product was obtained when pH>8 in comparison to acidic conditions in glucose-lysine, glucose-methionine, glucose-threonine model systems.

MR can occur at room temperature or at high temperatures [2]. This has also been explored in prior study by Hurrell and Carpenter [20],where a glucose-albumin model system was heated under different conditions (37 °C-30 day and 121 °C-15 min). According to that study, there was lysine loss after both heat treatments, but it was higher after processing at 121°C for 15 min, showing that the increase in thermal load accelerated

the MR [20]. As the temperature-time (thermal load) increases, the speed of MR rises. However, the important thing here is that while achieving inhibition of MR, the temperature and time should not be lowered below the limits that will not eliminate pathogens and enzymes. For this purpose, studies are being carried out on new heat treatment technologies that will ensure food safety with minimal thermal load [21].

The amount of water in the environment is one of the factors affecting MR. MR proceeds faster at water activity between 0.5 and 0.8. Rate of MR slows down at high water activity values because high water activity reduces the concentration of Maillard reaction intermediate products (MRIP), similarly, despite increased concentration of MRIP at low water activity values, the decrease in the mobility of MRIP slows the MR [12]. However, under low humidity conditions, MR is not completely blocked. Robert et al. [22] showed that mobility of reactants in the acrylamide formation were affected by carbon dioxide and ammonia formed in the intermediate steps of MR, as well as from the water that was released from the structure under low humidity conditions.

The type of free amino and carbonyl group is another factor affecting MR. Pentose sugars are more reactive than hexoses, hexoses are more prone to participate in MR than disaccharides, and aldose sugars are more reactive than ketose sugars [23,24]. Apolar and basic amino acids are more willing to participate in MR than acidic amino acids [24]. Additionally, α-dicarbonyl compounds which were formed from lipid oxidation and can react with free amino groups of amino acids even if there is no reducing sugar in the system [15], and to prevent lipid oxidation, removing oxygen from the system could be a good solution [2].

Kwak and Lim [24] reported that Fe^{+2} and Cu^{+2} ions in the environment accelerate MR, on the other hand, high concentration (10%) of NaCl acts to slow down the MR. In a publication on the impact of Ca^{+2} ions on the MR, acrylamide level in the product decreased with the addition of Ca^{+2} ions but the HMF level increased [25]. Sulphureous compounds are also known to inhibit MR reaction. However, they have undesirable sensory effects [2]. MR can also be prevented by phenolic compounds due to their antioxidant activity [26]. Based on these studies, the impact of minerals and other chemical compounds on MR depends on the process parameters and product matrix [26].

2.2. Acrylamide

MR, which is the result of heat treatment applied to foods, helps to improve the properties of foods such as taste, aroma, texture, and color, besides the formation of heat treatment contaminants with toxic properties such as acrylamide [27]. Since the 1950s, acrylamide has been synthesized artificially and used for many purposes such as polyacrylamide gel production, wastewater clarification, treatment of paper and in cosmetic industries [28,29]. In 1994, acrylamide was announced by the International Agency for Research on Cancer (IARC) as a harmful compound to human health and was added to list "Class 2 A" which is the list of probable carcinogens for human [30]. In 2002, Swedish National Food Administration declared that acrylamide was a toxic food-borne heat treatment contaminant [31] and acrylamide is announced to be formed by MR between reducing sugars and asparagine in intensively heat treated carbohydrate and asparagine-rich foods (Figure 1.6) [32]. Thus, it has been shown that acrylamide is not only produced by chemical synthesis, but also spontaneously forms as result of a heat treatment.

Acrylamide is absorbed in the gastrointestinal tract and participates to the blood circulation after being taken into the body with food consumption and enters the liver where it is detoxified and converted into glycidamide, a reactive genotoxic-mutagenic epoxide [33]. Additionally, acrylamide may conjugate with glutathione [34]. As a result of long-term exposure to acrylamide, even in small amounts, glycidamide disrupts the structure of DNA and the damages it which causes increased carcinogenic effects [35,36].

By detecting the formation of acrylamide in commonly consumed foods such as wheat flour-based products, French fries, and coffee [37], food scientists, food manufacturers and authorities have increased interest for acrylamide which also promoted the new studies on the issues related to the toxicity, metabolism, formation mechanism, and mitigation strategies of acrylamide [29].

The amount of acrylamide taken into the body daily varies depending on the person, country, process parameters of foods. The average daily acrylamide intake determined by Food and Agriculture Organization/World Health Organization (FAO/WHO) is 0.2-1 mg/kg body weight [28,38]. However, the amount of acrylamide detected in processed food products is much higher.

There is no clear limit or regulation regarding the content of acrylamide in foods but there are recommendations published by EFSA [26]. "Acrylamide Toolbox" is prepared by the authorities in order to mitigate the acrylamide in food, to prevent negative effects on human health after long exposure, to provide information and guidelines to help food producers about acrylamide reduction strategies [39].

2.2.1. Acrylamide Formation Mechanism

After it was determined that acrylamide is a harmful compound to human health found in processed foods, many studies were carried out to explain its formation mechanism. As a result of these studies, some major and minor reaction pathways were found even not all formation mechanisms were fully explained [40].

2.2.1.2. Major Pathways of Acrylamide Formation

One of the most important reaction routes responsible for the acrylamide formation is the MR between the carbonyl group in reducing sugars and the amino group in free asparagine during heat treatments (Figure 2.2) [41,42]. Asparagine is the most important precursor in acrylamide formation, and it has been reported that asparagine alone can also form acrylamide without the need to interact with any other compound, but the reaction rate is much lower than the rate of formation through MR [42,43].

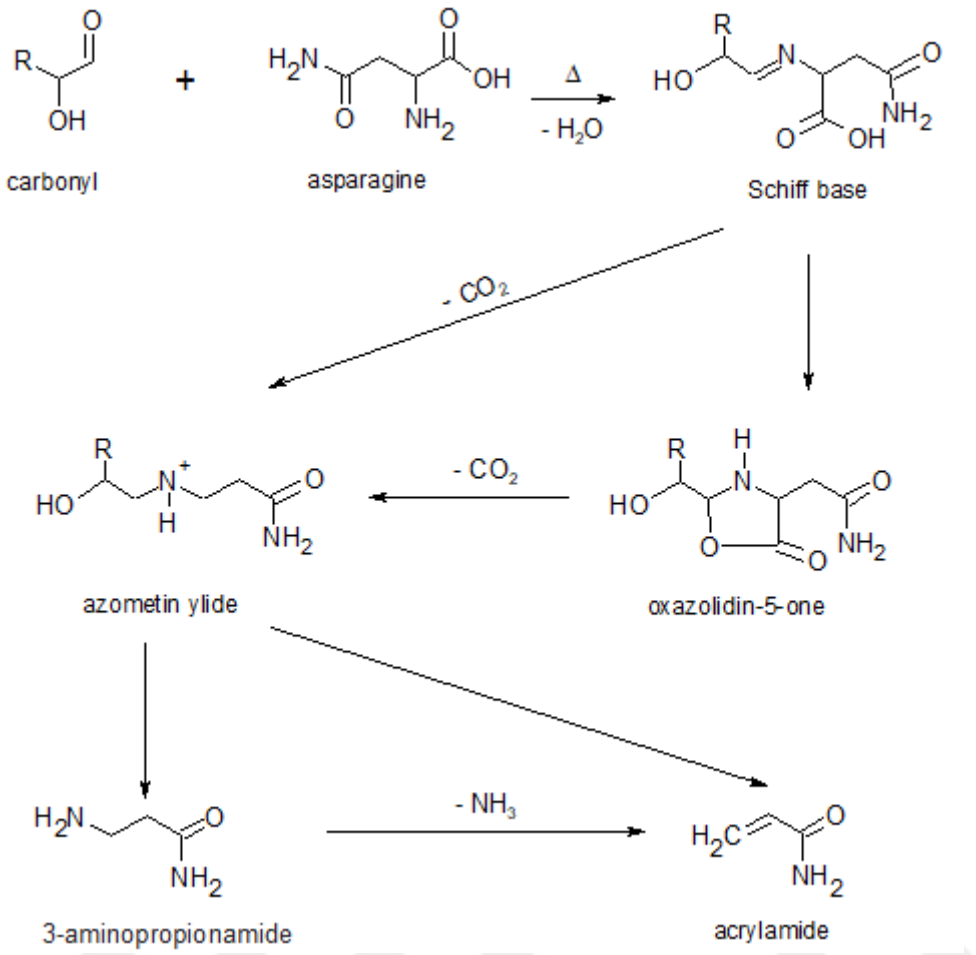


Figure 2.2. Acrylamide formation mechanism via Maillard reaction, adopted from [40].

In the initial stage of the MR responsible for the formation of acrylamide, the carbonyl group in reducing sugars interact with the amino group in the asparagine, and N-glycosyl-asparagine, the Schiff base, is formed [42]. In systems with low water content, Schiff base undergoes direct decarboxylation or decarboxylation via the intermediate product, oxazolidin-5-one, resulting the formation of azometine ylide [42,44]. Azometine ylide forms acrylamide by breaking it down directly or deaminating through the 3-aminopropionamide (3-APA) intermediate product [42]. Additionally, 3-APA produced by enzymatic decarboxylation of asparagine can be easily converted into acrylamide [33].

Amadori product can be formed from Schiff base, but acrylamide formation does not occur directly from the Amadori product [45]. It has been suggested that degradation of Amadori product to reactive carbonyl compounds contributes to the formation of acrylamide by reacting with asparagine [32].

2.2.1.3. Minor Pathways of Acrylamide Formation

There are many minor pathways of acrylamide formation (Figure 2.3). One of these formation mechanisms is through acrolein, a three-carbon aldehyde formed by lipid oxidation, carbohydrate degradation, or heating of glycerol at high temperatures [40]. At the initial stage of this reaction, acrolein is oxidized and acrylic acid is formed, and acrylic acid reacts with ammonia removed from the structure α-amino acids in SD, and finally, acrylamide is formed [40,46]. Limited amounts of acrylamide formation from acrolein is probably because of volatility of ammonia and its limited presence in the environment [40].

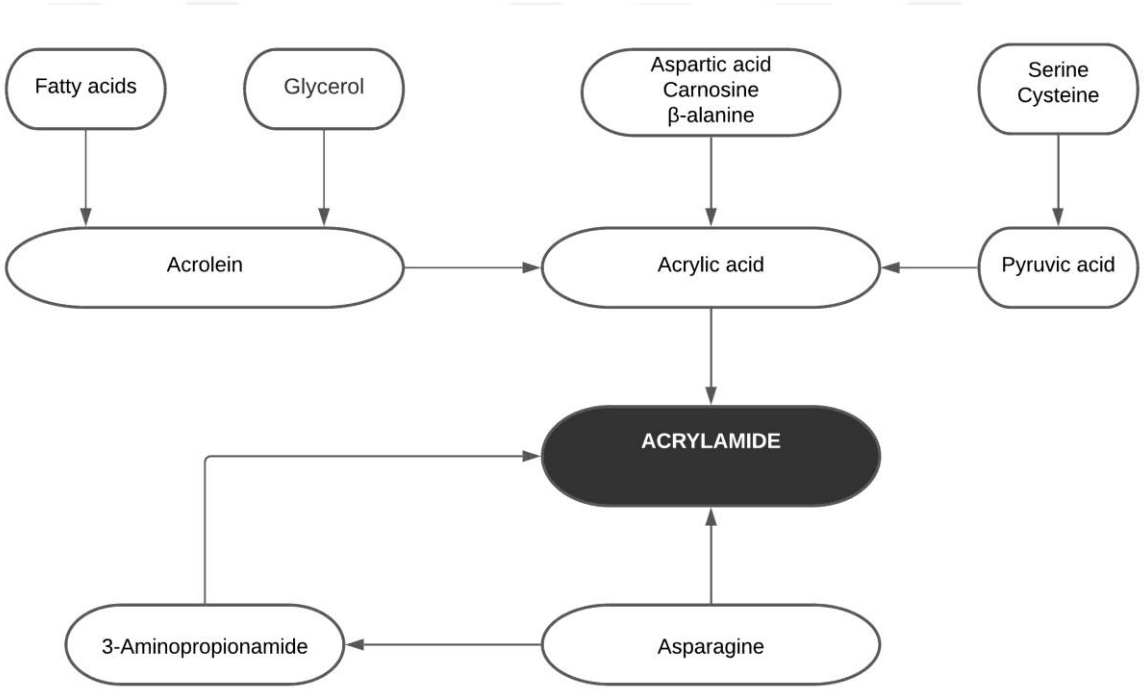


Figure 2.3. Minor pathways of acrylamide formation, adopted from [47].

The formation of acrylamide is also reported through pyruvic acid. At the end of the degradation of the cysteine and serine, pyruvic acid is formed and transformed to lactic acid. Acrylic acid formed by lactic acid dehydration reacts with ammonia in the environment and acrylamide is formed. It has also been reported that acrylamide can directly form carnosine, β-alanine and aspartic acid [48].

2.2.2. Factors Effecting the Formation of Acrylamide

There are many components and parameters in foods that affect the formation or inhibition of acrylamide, especially pH, thermal load, reducing sugar type, moisture content, and amino acid type [40,42,49].

2.2.2.1. Reducing Sugars

Reducing sugars are carbohydrates such as glucose, fructose, deoxyglucose, ribose, which have a carbonyl group that can easily give electrons and react with the amino groups in asparagine for the formation of acrylamide [49]. As the carbon skeletal length of the reducing sugar shortens, its compound structure becomes more tense, making the carbonyl group more open to interaction, attacking the amino group more, causing more acrylamide formation [42]. Additionally, sucrose which is a non-reducing sugar, and polymers such as starch, break down into their monomers under acidic conditions when the temperature exceeds 100 ºC, and react with asparagine to form acrylamide [49].In a study conducted by Becalski, Lewis, and Seaman [50], asparagine was heated with sucrose, glucose and fructose in separate models, and fructose was found to be the most reactive sugar while sucrose was the least reactive. It was reported that there is a positive interrelation between the increase in the total amount of sugar and the formation of acrylamide [49].

2.2.2.2. Amino Acids

The only amino acid that directly forms acrylamide is asparagine although amino acids such as cysteine, serine, and aspartic acid are known to contribute indirectly to the formation of acrylamide [42,43,48]. Therefore, high free asparagine content of products such as potato-based products (40% of the total free amino acid content) and wheat flour-based products (14% of the total free amino acid content) are highly suitable for acrylamide formation [32]. Additionally, it was reported that there is a correlation between the increase of the total amount of amino acids in wheat-based products and the formation of acrylamide, but the total amount of amino acids in potato-based products has no effect on acrylamide formation [49].

2.2.2.3. Lipids

Lipid rich food matrices are susceptible to the formation of carbonyl compounds including α-dicarbonyl compounds such as glyoxal and methylglyoxal, these carbonyl compounds promote the formation of acrylamide [15].

2.2.2.4. Moisture Content and Water Activity

Acrylamide formation generally occurs on a dry surface and maximum acceleration of acrylamide formation is at moisture content of 10–20% at bakery products. However, the content of acrylamide remains constant as acrylamide degradation can also occur in environments with a lower water content [49]. The most efficient acrylamide formation occurs at 0.3-0.7 water activity values [51].

2.2.2.5. pH

The pH of the food matrix controls the formation of acrylamide by directing the MR progression path. Acrylamide formation prefers alkaline conditions (maximum at pH 8), and this is due to the fact that the amino group in the asparagine is more willing to interact with carbonyl groups by protonation [52]. The decrease in pH prevents the protonation of the amino group, so restricting the formation of Schiff base hence acrylamide [49].

2.2.2.6. Thermal Load

Acrylamide formation increases at high temperatures and its formation on the surface of the food is greater than the center of the food because the temperature on the food surface can reach 100 ºC but core of the food matrix cannot [40]. However, when heat treatment is continued for a long time, the amount of acrylamide in the food matrix decreases [40]. Ryderbeg et al. [52], reported that acrylamide decreased in the potato model heated at 200 ºC for 60 min due to degradation of acrylamide and loss of water.

2.3. Acrylamide in Bakery Products

Bakery products are grain-based heat-treated products with carbohydrate, lipid, and protein content that allow rapid consumption. While bakery products can be classified under many subtitles, the most commonly produced types are hard biscuits with high water content (sheeted dough), hard biscuits with low water content (rotary dough), soft biscuits (wire-cut), pretzel-like snacks and wafers. One of the important terms in

classification is the short dough expression. Here, the dough is obtained by creaming method, the role of fat in dough formation is to compete with water and interrupt gluten development in flour added later to the system. Wire-cut dough and rotary dough are examples of such products. This type of short dough type products is processed mechanically more easily than products with developed gluten (hard dough) and expand a little during baking [53–55].

Sheeted dough has a soft dough structure with high moisture content and fat content. Generally, there is a risk of gluten development, since dough preparation is carried out using the all-in-one mixing method, where all ingredients are mixed at the same time. Protease enzyme is used to eliminate this risk [53–55].

Rotary dough is a type of product that has a low moisture content and high fat content, and a dough structure that crumbles easily. Since the creaming method is used for this dough preparation, there is no risk of gluten development. In this method, firstly, cream is obtained by mixing sugar and fat, then water-soluble ingredients are added to the medium, and flour is added at the last stage to complete the dough making. On the other hand, wire-cut dough, has a soft dough structure since its fat and sugar ratio is higher than other doughs. This dough type is prepared by the creaming method [53–55].

Pretzel-like snacks are a bakery product that has a dough prepared by the all-in-one mixing method, which is fermented or not, and is shaped by extrusion and baked on perforated bands [53,54].

Wafer dough is a dough that contains a very high amount of water (more than 50%) compared to biscuit dough and is prepared fluidly by the all-in-one mixing method. The fluid dough is baked between two hot plates. Wafer is generally low in enrichment with fat and sugar, and instead of being consumed alone, it is consumed with chocolate or cream [53,54].

As a result of the heat treatment, acrylamide formation also occurs in such foods and even if the formation of acrylamide is limited in some bakery products, excessive consumption of such products in nutrition is harmful to human health due to high acrylamide intake. [29].

European Commission published a recommendation in 2017 and 2019 (2017/2158/EU and 2019/1888/EU) to help to mitigate the presence of acrylamide in foods. The maximum amount of acrylamide that can be found in a food, which is the benchmark level, is thought that these acrylamide limitations in foods will become more and more legal and create a permanent regulation. EFSA asks food manufacturers to produce low acrylamide content food in accordance with these limitations and advises on the implementation of mitigation strategies it has created. According to this published regulation, the benchmark level for biscuits and wafers is 350 μg/kg, and the benchmark level for crackers with the exception of potato-based crackers is 400 μg/kg [56].

2.3.1. Mitigation Strategies of Acrylamide in Bakery Products

In many countries around the world, the daily acrylamide intake of bakery products is notable, and these rates of daily acrylamide intake are 40% in the United States of America, 24% in Sweden, 44% in Belgium, 25% in Germany, 14-37.5% in Romania [29,57]. Due to the contribution of bakery products in daily acrylamide intake, reduction of acrylamide in bakery products as well as other acrylamide-containing products in order to protect human health is essentially important [29]. There are some strategies to mitigate acrylamide in bakery products. These strategies are research-based, pilot-scale, or industry-ready, and they are generally categorized with 3 main headings: agronomy, recipe, processing (Figure 2.4) [38,39].

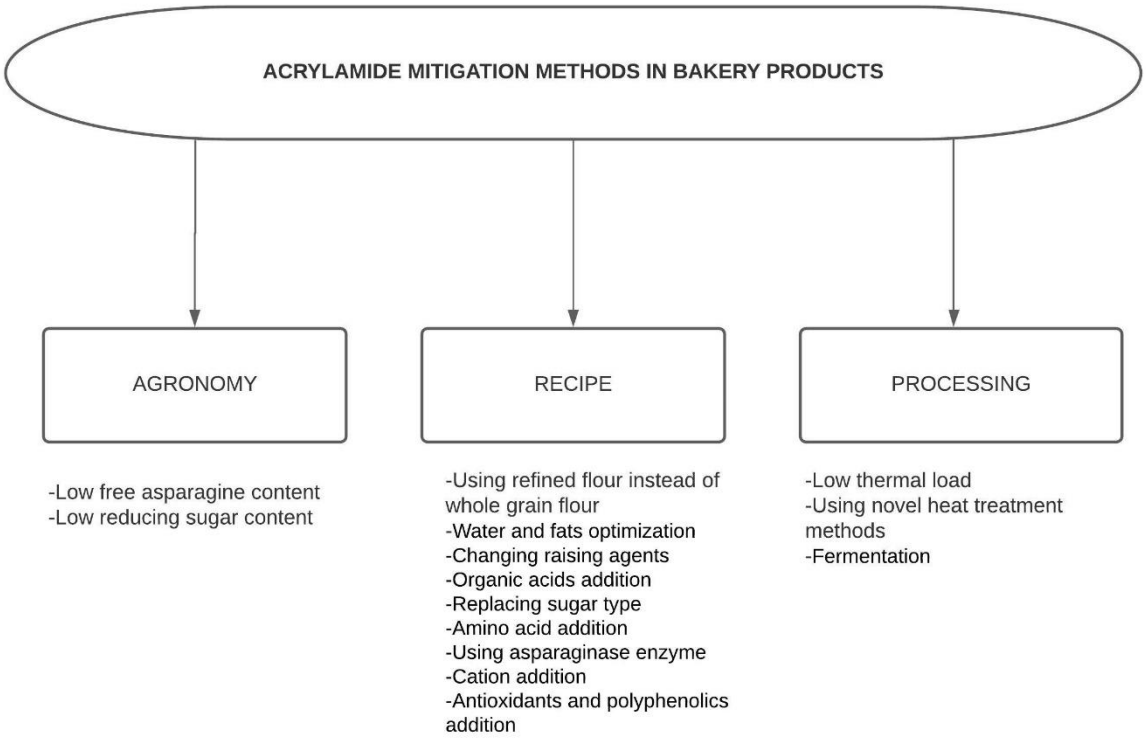


Figure 2.4. Acrylamide mitigation methods in bakery product.

2.3.1.1. Agronomy

Two of the important precursors that cause the formation of acrylamide in bakery products are presence of reducing sugars and the amount of asparagine. One of the agronomic practices is to develop species which have ability to synthesize less of such precursors during plant development. Another possible way to control acrylamide in plants is to control environmental conditions in order not to promote the formation of precursors [39]. For instance, 1-1.3% of the dry weight of soft wheat is reducing sugars, but actually the main precursor in acrylamide formation is asparagine [39]. The amount of free asparagine in grain varieties is affected by the climate, fungal disease, the amount of sulphur and nitrogen in the soil [29,39,57]. For example, the low amount of sulphur in the soil, the use of dense nitrogen fertilizer, and some fungal diseases increase the amount of free asparagine in cereals [26,38,39]. In addition, increasing the effectiveness of grain milling is another way of reducing asparagine, by removing the bran and germ parts which have high asparagine content, in flour [58].

2.3.1.2. Processing

As the thermal load applied to bakery products increases, acrylamide formation increases. For example, Van Der Fels-Klerx et al. [59], found that the highest acrylamide formation in biscuits subjected to heat treatment at 180 ºC, 190 ºC, 200 ºC for 8-15 minutes was in the biscuits processed at 200 ºC for 15 min.

The development of new heat treatment techniques that can be applied with lower thermal load compared to traditional methods helps manufacturers to mitigate acrylamide [26]. Since the only area where the temperature reaches 100 ºC in bakery products is the food surface, this is the place where acrylamide formation is most common [57]. The use of radio frequency heating method, instead of traditional heating, comes to the forefront as an effective method in reducing acrylamide by ensuring that thin products such as biscuits are heated quickly and the temperature is not raised too much although color intensity is low [26]. Anese et al. [60], found a 43% reduction in acrylamide by using vacuum in addition to traditional heat treatment during the cooking of biscuits.

Through the fermentation, both reducing sugar and asparagine are utilized by yeasts, thereby limiting the formation of acrylamide. In addition to that fermentation helps reduction of pH by formation of lactic acid which also reduces the acrylamide formation [39]. It was also reported that lactic acid formed as a result of fermentation or added lactic acid subsequently reduces the amount of acrylamide in rye bread [61].

2.3.1.3. Recipe

Ingredients in recipes, in which food products are prepared from, affect the acrylamide content [26]. Even whole-grain flour is beneficial to human health in terms of high nutritional and protein content, whole grains should not be used in foods that are asked to reduce acrylamide because of the high asparagine content in whole flours [39]. Capuano et al. [62], showed that acrylamide formation in bread crisp prepared from whole wheat flour (291 µg/kg) was higher than normal wheat flour bread crisp (262.3 µg/kg).

Water and fats affect both quality characteristics and acrylamide formation in bakery products [58]. Anese et al. [63], provided that in their study, increasing the amount of water in biscuits increases the formation of acrylamide, on the contrary, the increase in the amount of fat reduces the formation of acrylamide. They argued that the reason for

this situation is that water increases the mobility of the reactants, while the oil creates this effect by making it difficult for the reactants to come together. On the other hand, lipid oxidation products in lipid-rich products are also known to increase acrylamide formation [46].

Usage of raising agents, especially in biscuits, changes the amount of acrylamide in the product [39]. Ammonium bicarbonate (NH_4HCO_3), one of the most commonly used raising agents, increases the amount of α-dicarbonyl carbonyl compounds and also acrylamide by releasing ammonia to the product during heating. Using sodium bicarbonate ($Na_4HCO_{3)}$ instead of ammonium bicarbonate significantly reduces the amount of acrylamide [57]. Amrein et al. [64], have proven that there is a high positive interrelation between the usage of ammonium bicarbonate and acrylamide formation. A 60% reduction in acrylamide occurs when sodium bicarbonate is used instead of ammonium bicarbonate as a raising agent in gingerbread. However, ammonium bicarbonate is recommended to be used in combination with other sodium bicarbonate, as the use of only sodium bicarbonate causes difficulty in achieving the desired texture properties of the product [39].

Increasing acidity in bakery products by adding organic acid reduces the formation of acrylamide by changing the MR pathway against acrylamide formation [39]. Amrein et al. [64], found that addition of different concentrations of citric acid to the gingerbread recipe reduced acrylamide by about 4-40 times compared to the control. However, the decrease in pH caused the formation of other harmful compound such as HMF and resulted in lighter color [39].

The usage of glucose or sucrose, instead of fructose, reduces the acrylamide content in a limited way, but problems occur in terms of aroma of the end product [39]. It was shown that the use of sucrose instead of reducing sugar resulted in 20 times reduction in acrylamide [64].

The use of other types of amino acids provides a decrease in the formation of acrylamide in bakery products [39]. It was found that the formation of acrylamide was significantly mitigated but browning intensity increased, when glycine amino acid was added to gingerbread [64]. The addition of lysine to the dough both increases the nutritional value

of the dough in terms of the essential amino acids contents and limits the formation of acrylamide, however darker end products are obtained [57].

Divalent cations are another factor that affects the formation of acrylamide [29]. Açar et al. [25], added different concentrations of $CaCl_2$ to the cookies in their study, as a result of which the amount of acrylamide decreased. However, on the contrary to the reduction of acrylamide, the amount of HMF increased. Possible reason for this situation is that Ca^{+2} ions increase the interaction between asparagine and the matrix and give high thermal stability also prevent them from reacting with α-dicarbonyl compounds [65].

Many studies have found that the supplementation of antioxidants and polyphenolic compounds to bakery products helps to reduce acrylamide formation [66,67]. For example, 57-62% acrylamide reduction was observed in wheat buns by the addition of antioxidants from rosemary leaves to dough [67]. However, there are studies contradictory to the reduction of acrylamide by addition of antioxidant compounds [68]. As a result, the effect of antioxidant and polyphenolic compounds on acrylamide formation varies according to the food matrix [36].

One of the most effective acrylamide mitigation strategies in baking products is the usage of the enzyme ‘asparaginase’. The asparaginase can reduce acrylamide formation very efficiently by transforming asparagine, the main acrylamide precursor in bakery products, into aspartic acid [39]. However, cost of enzyme, pH of the product, recipe of the product, and process conditions affect enzyme activity [39].

2.4. Acrylamide Mitigation By L-Asparaginase

Many strategies for acrylamide mitigation in bakery products continue to be developed and implemented, however, some of these methods cause significant losses in the flavor, color, and texture [69]. Additionally, the use of some acrylamide reduction strategies makes it necessary to change the formulation of the product or the process parameters [27]. The use of the L-asparaginase as an acrylamide reduction strategy comes to the forefront as it hydrolyzes asparagine to aspartic acid without causing any loss in the sensory quality of the product [29,39].

L-asparaginase (E.C.3.5.1.1) is an enzyme belongs to the amino acid amidohydrolase enzyme class obtained from some bacteria, fungi [70], yeast, and green plants [71]. L-asparaginase was used only for curing of leukemia at past years but with the realization of the importance of L-asparaginase in reducing acrylamide formation, L-asparaginase production studies and its use in the industry began to increase and the L-asparaginase market value reached to 380 million USD in 2017 [72]. U.S. Food and Drug Administration (FDA) has included only L-asparaginase produced from *Aspergillus oryzae* and *Aspergillus niger* in the GRAS (Generally Recognized as Safe) category and allowed its commercial use for the food industry [38,39]. There is no ban on the use of commercial L-asparaginase, which does not pose a problem for human health, primarily in the USA, EU countries, Russia, China, Canada and many other countries of the world [39].

The mechanism of the reaction of the L-asparaginase is the conversion of asparagine to aspartic acid by hydrolyzing the amine group of asparagine and removing ammonia from asparagine (Figure 2.9) [27,73]. Mitigation amount of asparagine, which is the main acrylamide precursor in bakery products, greatly limits the formation of acrylamide [74]. Although L-asparaginase is mostly asparagine specific and does not react with other amino acids, some L-asparaginase species react with glutamine on a limited basis, but this is undesirable because of its negative effects on human health [69].

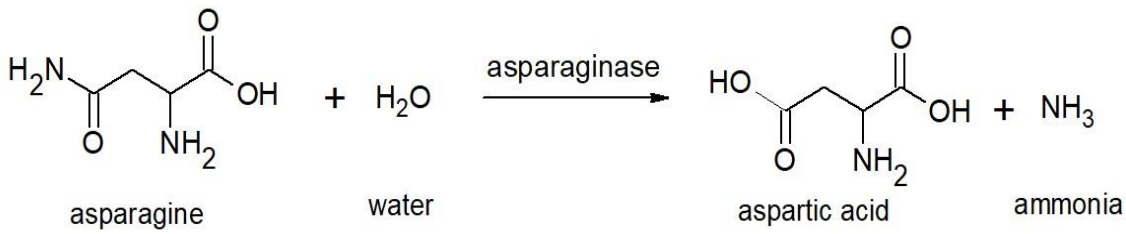


Figure 2.9. Mechanism of the L-asparaginase, adopted from [74].

There are many elements such as pH, temperature, amount of enzyme used, enzyme treatment time, dough resting time, amount of water, amount of fat, and homogeneous mixing of ingredients that affect the efficiency of application of L-asparaginase in bakery product [39,63,73]. Additionally, there are some factors that limit the widespread use of L-asparaginase in the food industry for now. These are the high cost of the enzyme, the

need of research for the determination of the optimum enzyme dosage, the determination of most effective temperature, pH, enzyme processing time, stopping enzyme activity during heat treatment and lack of continuous process [63,73]. High cost of the enzyme may be compensated by increasing L-asparaginase application in food industry and by reducing time necessary for enzyme application [69].

There are two products on the market which are PreventASe™ from DSM (Heerlen, Netherlands) and Acrylaway® from Novozymes A/S (Bagsvaerd, Denmark). They are produced from microbial sources and allowed to be used as commercial L-asparaginase [69]. PreventASe™, which was offered the first for sale by DSM in 2007, is produced with recombinant DNA technology from *Aspergillus niger* and its optimum operating parameters are pH 4-5 and 50 °C [69]. Acrylaway® produced by Novozymes A/S from *Aspergillus oryzae* can show activity in more basic conditions and at lower temperatures than PreventASe™ (optimal pH 7 and 37 °C) [69]. In calculating the activity of Acrylaway®, spectrophotometric method is used for the quantitative analysis of ammonia produced from asparagine by the effect of L-asparaginase, interacting with α-ketoglutarate to form L-glutamic acid. Then, the data obtained as a result of the analysis and ASNU (amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions) is used for the activity of Acrylaway® [69].

The number of recent studies on the use of these two commercial enzymes in the field of bakery products (especially biscuits, crackers, and wafers) is very limited. Additionally, the number of studies on its use in industry or on a pilot scale is negligible [69]. Also, for the use of such L-asparaginase, there is a need for studies on process stabilization and their allergic effects [75].

3. MATERIALS AND METHODS

3.1. Chemical and Consumables

The flour and shortening, potassium metabisulfite, invert sugar, crystal sugar, sodium hydroxide, ammonium bicarbonate, sodium bicarbonate, sodium acid pyrophosphate lecithin, NaCl needed to prepare the dough were obtained from a local market. Acrylamide (99%), asparagine (98%), potassium hexacyanoferrate (III), zinc sulfate, acetonitrile (gradient grade), were purchased from Sigma (Deisenhofen, Germany). The Carrez I solution was obtained by dissolving 15 g potassium hexacyanoferrate (III) in 100 mL of deionized water. Carrez II solution was obtained by dissolving 30 g zinc sulfate in 100 mL of deionized water. Commercial asparaginase enzyme with an activity of 3500 ASNU/g was obtained from a foreign company. Syringe filters (nylon, 0.45 μm), pH calibration solutions at pH 4 and 7 were from IsoLab (Wertheim, Germany). Oasis MCX cartridges were obtained from Waters (Millford, MA, USA). The SeQuant ZIC-HILIC (150 x 4.6 mm, 3.5 μm) column was purchased from Merck (Darrmstadt, Germany) and Atlantis C18 (150 mm × 4.6 mm, 5 μm), was purchased from Waters (Millford, MA, USA). Deionized water was used though out the experiments (0.55 μS/cm). Formic acid (98%) and methanol were obtained from Merck (Darmstadt, Germany). Deuterated acrylamide was purchased from Toronto Research Chemicals Inc. (North York, Canada).

3.2. Preparation of the Dough and Baking

Hard biscuits with high water content (sheeted dough), hard biscuits with low water content (rotary dough), and soft biscuits (wire-cut) was prepared according to Manley [53],with some modifications. However, the biscuits recipes were not disclosed here as they were purchased from a local company and are a trade secret. Additionally, pretzel-like snack dough and wafer dough were prepared according to the recipe given in elsewhere [53].

All in one mixing method was used for the preparation of sheeted dough and all ingredients were added to mixer (Kitchen aid artisan mixer, Benton Harbor, MI, USA) and mixed for 12 min in fast mixing mode (4 speed level) to prepare the sheeted dough (control). To increase the efficiency of the asparaginase in reducing the formation of acrylamide, different types of applications were performed. These applications were

increasing the enzyme dosage, the dough resting time and/or temperature, the mixing time and speed, and adding the shortening to the mixing medium after a period of mixing time. Enzyme treatments for sheeted dough were 1000 ASNU: The dough containing 1000 ASNU/kg flour, 2000 ASNU: The dough containing 2000 ASNU/kg flour, 3000 ASNU: The dough containing 3000 ASNU/kg flour, 2000 ASNU + S: The shortening was added to the dough containing 2000 ASNU/kg flour after 8 min of mixing, 1000 ASNU + 15 Min RT: The dough containing 1000 ASNU/kg flour was rested for 15 min at 25 °C, 2000 ASNU + 15 Min RT: The dough containing 2000 ASNU/kg flour was rested for 15 min at 25 °C, 3000 ASNU + 15 Min RT: The dough containing 3000 ASNU/kg flour was rested for 15 min at 25 °C, 2000 ASNU + 15 Min x 37 °C RT: The dough containing 2000 ASNU/kg flour rested for 15 min at 37 °C, 2000 ASNU + MS: The dough containing 2000 ASNU/kg flour mixed at high speed (level 10) for 12 min, 2000 ASNU + MT: The dough containing 2000 ASNU/kg flour mixed at medium speed (level 4) for 15 min, 2000 ASNU+ MS x MT: The dough containing 2000 ASNU/kg flour mixed at high speed (level 10) for 15 min, 2000 ASNU + 30 Min RT: The dough containing 2000 ASNU/kg flour was rested for 30 min at 25 °C. The dough was sheeted between two baking papers to a thickness of 2 mm and the biscuits were cut by using a round shape cutter with a diameter of 44.5 mm. A total of 6 biscuits were placed to a conventional oven (Memmert UN 55, Schwabach, Germany) and baked at 230 °C for 8.5 min with a 100% flap on.

Creaming mixing was used in hard biscuits with low water content (rotary dough) and soft biscuits (wire-cut). In this method, first the cream was formed by mixing shortening and sugar, (2 speed level – 4 min mixing) then water-soluble ingredients were added (2 speed level – 4 min mixing), and in the last step, flour was added together with the asparaginase enzyme to make the dough (control) (2 speed level – 2 min mixing). The mixing speed was moderate compared to sheeted dough. To increase the efficiency of the enzyme asparaginase in reducing the formation of acrylamide; the enzyme dosage, the dough resting time and/or temperature was increased, and mixing regime was changed.

For rotary dough application, the enzyme treatments were, 2000 ASNU: The dough containing 2000 ASNU/kg flour, 2000 ASNU + 15 Min RT: The dough containing 2000 ASNU/kg flour was rested for 15 min at 25 °C, 2000 ASNU + 15 Min x 37 °C RT: The dough containing 2000 ASNU/kg flour was rested for 15 min at 37 °C, 2000 ASNU +

$NaHCO_3$: The sodium bicarbonate was added to the dough containing 2000 ASNU/kg flour after creaming stage, 2000 ASNU + MOC: The water soluble ingredient mixture and flour containing 2000 ASNU/kg flour were mixed, then the prepared cream was added to make the dough, 5000 ASNU+ 30 Min RT: The dough containing 5000 ASNU/kg flour was rested for 30 min at 25 °C. The thickness and diameter of the molded dough was the same as the sheeted dough. The baking was performed at 210 °C for 8.5 min with a 100% flap on conditions and a total of 6 biscuits was placed to the oven in each baking.

For wire-cut biscuits, 1000 ASNU: The dough containing 1000 ASNU/kg flour, 2000 ASNU: The dough containing 2000 ASNU/kg flour, 3000 ASNU: The dough containing 3000 ASNU/kg flour, 5000 ASNU: The dough containing 5000 ASNU/kg flour, 7000 ASNU: The dough containing 7000 ASNU/kg flour, 9000 ASNU: The dough containing 9000 ASNU/kg flour, 12000 ASNU: The dough containing 12000 ASNU/kg flour, 1000 ASNU + 15 Min RT: The dough containing 1000 ASNU/kg flour was rested for 15 min at 25 °C, 2000 ASNU + 15 Min RT: The dough containing 2000 ASNU/kg flour was rested for 15 min at 25 °C, 2000 ASNU + 30 Min RT: The dough containing 2000 ASNU/kg flour was rested for 30 min at 25 °C, 3000 ASNU + 15 Min RT: The dough containing 3000 ASNU/kg flour was rested for 15 min at 25 °C, 5000 ASNU + 15 Min RT: The dough containing 5000 ASNU/kg flour was rested for 15 min at 25 °C, 7000 ASNU + 15 Min RT: The dough containing 7000 ASNU/kg flour was rested for 15 min at 25 °C, 9000 ASNU + 15 Min RT: The dough containing 9000 ASNU/kg flour was rested for 15 min at 25 °C, 9000 ASNU + 15 Min x 37 °C RT: The dough containing 9000 ASNU/kg flour was rested for 15 min at 37 °C, 12000 ASNU + 15 Min RT: The dough containing 12000 ASNU/kg flour was rested for 15 min at 25 °C, 2000 ASNU + MOC: The water soluble ingredient mixture and flour containing 2000 ASNU/kg flour were mixed, then the prepared cream was added to make the dough, 5000 ASNU + MOC: The water soluble ingredient mixture and flour containing 5000 ASNU/kg flour were mixed, then the prepared cream was added to make the dough, 9000 ASNU + MOC: The water soluble ingredient mixture and flour containing 9000 ASNU/kg flour were mixed, then the prepared cream was added to make the dough, applications were used.

The diameter and thickness of the wire-cut dough was the same as the sheeted dough and rotary dough. The 6 pieces of wire-cut dough were placed on the baking tray and baked at 210 °C for 8 min with a 100% flap, always in the same area of the oven.

In the production of pretzel-like snack dough, all in one mixing method was used. The ingredients were biscuit flour (100 g), NaCl (2.4 g), shortening (17.6 g) ammonium bicarbonate (1 g), water (32.4 g), potassium metabisulfite (0.09 mg) [53]. Formulation of pretzel-like snack is like pretzel dough. The ingredients were mixed after addition of all ingredients at once and a moderate mixing speed (4 speed level) was used for 3 min (control). To reduce acrylamide applied enzyme treatment were 2000 ASNU: The dough containing 2000 ASNU/kg flour, 3000 ASNU: The dough containing 3000 ASNU/kg flour, 2000 ASNU + 15 Min RT: The dough containing 2000 ASNU/kg flour was rested for 15 min at 25 °C, 3000 ASNU + 15 Min RT: The dough containing 3000 ASNU/kg flour was rested for 15 min at 25 °C, 2000 ASNU + 15 Min x 37 °C RT: The dough containing 2000 ASNU/kg flour was rested for 15 min at 37 °C.

In pretzel-like snack production, pasta production apparatus of the mixer was used and the pretzel-like snack sticks having the same diameter (2 mm) and length (80 mm) were obtained. A total of 24 pretzel-like snack sticks were placed on the baking tray by allowing same spaces between them. NaOH solution (1%, w/v) was sprayed on the surface of the pretzel-like snack sticks to provide a shiny surface. Then, the pretzel-like snacks baked at 210 °C for 9 min.

A basic wafer dough was prepared by using biscuit flour (100 g), NaCl (0.25 g), water (137 g), shortening (2.25 g), lecithin (1 g), sodium bicarbonate (0.32 g) according to recipe shown by the Manley [53]. Enzyme treatment were 1000 ASNU: The dough containing 1000 ASNU/kg flour. 2000 ASNU: The dough containing 2000 ASNU/kg flour. 1000 ASNU + 15 Min RT: The dough containing 1000 ASNU/kg flour was rested for 15 min at 25 °C. 2000 ASNU + 15 Min RT: The dough containing 2000 ASNU/kg flour was rested for 15 min at 25 °C. 2000 ASNU + 30 Min RT: The dough containing 2000 ASNU/kg flour was rested for 30 min at 25 °C. 1000 ASNU + 15 Min x 37 °C RT: The dough containing 1000 ASNU/kg flour was rested for 15 min at 37 °C. All in one method was used to produce wafer dough. The wafer dough was only evaluated in terms of its acrylamide formation potential by measuring asparagine.

In applications with a resting period, the dough was rested by wrapping it with a stretch film as bulk. A part of dough was placed into freezer at -40 °C to stop asparaginase activity as soon as the dough was prepared for further analysis. All baking experiments were performed twice. The samples taken out from the oven were cooled at room temperature for half an hour and were stored at room temperature after being packed for further analysis.

3.3. Measurement of Spread Ratio

The diameter and height of the biscuit samples were measured from different points 3 times by using a digital caliper, and the spread ratio was calculated by the ratio of diameter to height.

3.4. Analysis of Color

Color analysis of biscuits and pretzel-like snacks were performed by using a computer aided image analysis method previously identified by Mogol and Gökmen [76]. The photographs of the samples placed were taken on a dark background in an environment with equal light from all directions and analyzed in Matlab (The MathWorks, MA, USA). The two of the samples in each cooking set were selected, and two photographs of each were taken. L*, a* and b* describe different color tones, lightness, redness, and yellowness, respectively.

3.5. pH Measurement

The 10 mL portion of deionized water was added onto the frozen dough sample (1 g), which was stored at -40 °C until the analysis, and the mixture was vortexed in a shaker (Heidolph, Schwabach, Germany) for 15 min. Then, the mixture was centrifuged at 6080×g for 3 min in a centrifuge (Hettich Universal 320, Massachusetts, USA). After centrifugation, the fat layer on the supernatant were removed from the tube, and the pH of the clear supernatant was measured with a pH meter (Mettler & Toledo S220-K SevenCompact, Greifensee, Switzerland) previously calibrated by using the calibration solutions at pH 4 and 7. Measurement was made from each baking parallel.

3.6. Analysis of Water Activity

The dough sample was loaded into the cell of a water activity measurement device (Novasina LabTouch-aw meter, Lachen, Switzerland) immediately after preparation. Water activity was analyzed at 27°C and the two measurements were performed for each dough (control).

3.7. Analysis of Acrylamide

The extraction and analysis of acrylamide in biscuit and pretzel-like snack products were performed previously described by Gökmen et al. [77], with some modifications. A triple stage extraction procedure was followed to increase the extraction efficiency. A 10 mM formic acid solution containing 20 µg/kg of d3-acrylamide was prepared as the extraction solution. One gram of ground sample was weighed into a 15 mL falcon tube. Then, 0.5 mL Carrez I, 0.5 mL Carrez II, and 9 mL extraction solution were added onto the sample. The tube was vortexed for 4 min. After this step, the sample was centrifuged at 6080×g for 3 min and the supernatant was taken into a 50 mL falcon tube. In the second extraction step, 5 mL of extraction solution was added onto the pellet and the tube was shaken in the shaker for 4 min. Then, the tube was centrifuged at 6080×g for 3 min and the supernatant in the tube was taken into the 50 mL falcon tube. The operations in the third extraction step were the same as in the second step. After extraction, a part of the combined supernatant was transferred to an Eppendorf tube and freezed at -40 °C.

The Eppendorf tube was centrifuged at 10000×g for 3 min after thawing and the clear supernatant was passed through a preconditioned Oasis MCX cartridge. The preconditioning was performed by passing 1 mL water, 1 mL methanol and approximately 0.5 mL air through the cartridge. Before collecting the clear supernatants into a vial, the first 8 drops which passed through the cartridge were discarded.

Pure extracts collected into vials were analyzed using Waters Acquity H Class UPLC system (Millford, MA, the USA) connected to a triple quadrupole mass detector with electrospray ionization. Atlantis T3 column (4.6 x150 mm, 3µm) was used for the analysis. 10 mM formic acid in water was used as the mobile phase in the system at a flow rate of 0.2 mL/min at 25 °C. The electrospray source had the following properties: capillary voltage of 2.00 kV; cone voltage of 22 V; extractor voltage of 4 V; source temperature of 120 °C; desolvation temperature of 400 °C; and desolvation gas (nitrogen)

flow of 900 L/h. The flow rate of the collision gas (argon) was set to 100 L/h. Quantification of acrylamide was performed by using the fragmentation ion (m/z 55) of the precursor ions of m/z 72. Quantification of d3 acrylamide was performed by using the fragmentation ion (m/z 58) of the d3-acrylamide (m/z 75). The calibration curve was prepared at the level of 1, 2, 5, 10, 20 µg/L and extraction solvent was injected as blank. The results were reported as µg acrylamide/kg sample.

3.8. Analysis of Asparagine

Analysis of asparagine was performed according to Kocadağlı, Özdemir, and Gökmen [78] with some modifications. The extraction procedure was done in triple stages. An extraction solution containing 0.1% formic acid in acetonitrile:water (1:1, v/v) was prepared to both extract the free asparagine from the matrix and to stop the asparaginase activity. In the first step of the extraction, 10 mL extraction solution was added onto the dough (1 g) as soon as the dough was taken out from the freezer. Then, the content of the tube was mixed for 20 min until to obtain a homogenous slurry. After then, the tube was centrifuged for 6080×g for 3 min and the supernatant was transferred to a 50 mL falcon tube. In the second stage of the extraction, 5 mL of extraction solution was added onto the pellet and the sample was vortexed for 5 min. Then, the sample was centrifuged at 6080×g for 3 min and the supernatant was transferred into the 50 mL falcon tube. The third stage of the extraction was the same as the second extraction step. The combined extract was transferred to an Eppendorf tube and centrifuged at 10000×g for 3 min. The clear extract was diluted 1:1 by using acetonitrile and centrifuge at 10000×g for 3 min. Then, the supernatant was collected into a vial after passing through a 0.45 µm nylon filter.

The asparagine content of the samples was analyzed by Waters Acquity H Class UPLC system (Millford, MA, the USA) coupled with a triple quadrupole mass detector with electrospray ionization. SeQuant® ZIC®-HILIC column (150 x 4.6 mm, 3.5 µm) was used for the analysis. The mobile phases were 0.1 % formic acid in water (A) and %0.1 formic acid in acetonitrile (B). A gradient programme was applied which was 20:80 (A:B) for 2 min, 50:50 (A:B) for 5 min, 20:80 (A:B) for 3 min. The total run time was 10 min and the flow rate was 0.5 mL/min. The column temperature was 40°C. The electrospray source had the following properties: capillary voltage of 3.5 kV; cone voltage of 20 V; extractor voltage of 3 V; source temperature of 120 °C; desolvation temperature of 370

°C; and desolvation gas (nitrogen) flow of 900 L/h. The quantification of asparagine was performed by using the fragmentation of the precursor ion of m/z 133 to *m*/*z* 116. The asparagine standards used for the calibration curve were prepared at the level of 0.1, 0.2, 0.5, 1, 2 mg/L and extraction solvent was injected as blank. The results were given as mg asparagine/kg sample.

3.9. Statistical Analysis

All data were subjected to one way ANOVA analysis of variance and Tukey's test using SPSS version 22. [79] statistical program to reveal the similarities and differences between samples in a meaningful way in all experimental results. Differences at $p < 0.05$ were considered significant.

4. RESULTS AND DISCUSSION

4.1. Effect of Asparaginase Treatments on Acrylamide Reduction in Bakery Products and Asparagine Reduction in Their Dough

4.1.1. Effect of Asparaginase Treatments on Acrylamide Reduction in Sheeted Dough Biscuits and Asparagine Reduction in Sheeted Dough

Addition of asparaginase enzyme applied to bakery product doughs, it is expected that acrylamide formation will be limited due to asparagine reduction in sheeted dough.

Table 4.1. Acrylamide content (µg/kg sample) and acrylamide reduction (%) in sheeted dough biscuits after asparaginase enzyme treatments.

Treatments in sheeted dough biscuits	Acrylamide (µg/kg sample)	Acrylamide reduction (%)
Control	1672 ± 74^{a}	
1000 ASNU	1086 ± 29^{b}	35
2000 ASNU	588 ± 32^{de}	65
3000 ASNU	330 ± 42^{f}	80
1000 ASNU + 15 Min RT	933 ± 2^{bc}	44
2000 ASNU + 15 Min RT	398 ± 42^{ef}	76
3000 ASNU + 15 Min RT	67 ± 16^{g}	96
2000 ASNU + 30 Min RT	404 ± 47^{ef}	76
2000 ASNU + 15 Min x 37 °C RT	262 ± 103^{fg}	84
2000 ASNU + MS	790 ± 76^{cd}	53
2000 ASNU + MT	720 ± 16^{cd}	57
2000 ASNU+ MS x MT	774 ± 126^{cd}	54
2000 ASNU + S	820 ± 14^{cd}	51

*The same lowercase letters following the mean values indicate no statistical difference ($p<0.05$) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, S: Shortening, RT: Resting Time, MS: Mixing Speed, MT: Mixing Time.

The acrylamide concentration and acrylamide reduction in sheeted dough biscuits after enzyme treatments were given in Table 4.1. In 1000, 2000, and 3000 ASNU treatments, 35%, 65%, and 80% acrylamide reduction was achieved, respectively. As the enzyme dosage applied to sheeted dough was increased, the formation of acrylamide decreased significantly ($p<0.05$). 3000 ASNU treatment was the most effective enzyme treatment compared to 1000 and 2000 ASNU treatments with 330 ± 42 µg acrylamide/kg sample.

This acrylamide level was below the benchmark acrylamide level in biscuits which is 350 µg acrylamide/kg sample [56]. The reason for this effective asparagine reduction was that when the amount of enzyme was increased although the amount of substrate was constant, more asparagine turned into aspartic acid and the formation of acrylamide decreased.

To reveal the effect of resting on the asparaginase enzyme treatment in sheeted dough biscuits, treatments containing asparaginase enzyme with different dosages, but the same resting time (15 min) were applied (Table 4.1). There was no statistically significant difference between the acrylamide content of 1000 ASNU + 15 Min RT (933 ± 2 µg acrylamide/kg samples) treatment and 1000 ASNU treatment (1086 ± 29 µg acrylamide /kg samples) (p>0.05). The difference between the acrylamide content of the sheeted dough biscuits after 2000 ASNU + 15 Min RT treatment (398 ± 42 µg acrylamide/kg sample) and 2000 ASNU treatment to the dough (588 ± 32 µg acrylamide /kg sample) was not significant (p>0.05). Adding a resting period did not affect the enzyme-substrate interaction for the dosages of 1000 and 2000 ASNU. More acrylamide reduction was obtained in 3000 ASNU + 15 Min RT treatment (96%) compared to 3000 ASNU treatment (80%), and there was a statistically significant difference between the acrylamide reductions of these applications (p<0.05). This difference clearly indicated that when the appropriate dosage for the biscuit formulation was achieved, the enzyme able to reduce acrylamide and when resting was applied to the dough, further acrylamide reduction could be achieved due to the increased interaction time of enzyme and substrate.

In a study by Kukurova et al. [80], asparaginase enzyme (500 U/kg flour) was added to cookie dough and the dough without resting and with 15 min resting time was baked at 205 °C for 11 and 15 min. They found that acrylamide reduction was higher when the dough rested compared to the cookies prepared by without resting the dough at both baking conditions.

To observe the effect of prolonging the resting time on asparaginase application in sheeted dough biscuits (Table 4.1), 2000 ASNU + 15 Min RT and 2000 ASNU + 30 Min RT treatments were applied to the dough, respectively. The same acrylamide reduction levels were obtained for both 2000 ASNU + 15 Min RT (76%) and 2000 ASNU + 30 Min RT (76%) (p>0.05). This showed that the increase in the resting time has no effect on acrylamide reduction as 15 min already enough for the contact of enzyme and substrate.

Since 3000 ASNU/kg flour treatment was below the benchmark level in the sheeted dough biscuits, 2000 ASNU/kg flour dose was chosen to observe the effect of resting temperature on asparaginase treatments. As a result, it was revealed that there was no statistically significant difference in the acrylamide content of sheeted dough biscuits after 2000 ASNU + 15 Min x 37 °C RT (262 ± 103 µg acrylamide/kg sample) and 2000 ASNU + 15 Min RT treatment to the dough (398 ± 42 µg acrylamide kg sample) (Table 4.1) (p>0.05). However, when the acrylamide levels were compared, 2000 ASNU + 15 Min x 37 °C RT treatment to dough reduced the acrylamide of biscuits below the benchmark level although acrylamide was higher than the benchmark level after 2000 ASNU + 15 Min treatment.

2000 ASNU + MS, 2000 ASNU + MT, 2000 ASNU + MS x MT treatments were applied to the dough to test the effect of increasing mixing time and speed on asparaginase, respectively. It was found that 2000 ASNU + MS, 2000 ASNU + MT, 2000 ASNU + MS x MT treatments applied to dough reduced the acrylamide content of sheeted dough biscuits 53%, 57% and 54%, respectively. However, the absence of a significant difference between these reduction levels (p>0.05) and the 2000 ASNU reduction level indicated that mixing speed and time do not have a reducing effect on acrylamide formation.

To understand the effect of fat on the interaction of enzyme and substrate, first the ingredients other than shortening was mixed for 8 min and then shortening incorporated to the dough. As a result, there was not statistically significant difference between the acrylamide content of the biscuits after 2000 ASNU + S and 2000 ASNU treatments of the dough, respectively (p>0.05). In the literature the presence of fat in the food matrix limited the association between enzyme and substrate and using hydrogel instead of fat allowed easier interaction of enzyme and substrate in the food matrix [63]. This result showed that, on contrary to expectations, the shortening did not have a negative effect on enzyme and substrate interaction.

Table 4.2. Asparagine concentration (mg/kg dough) and asparagine reduction (%) in sheeted dough biscuits after asparaginase enzyme treatments.

Treatments in sheeted dough biscuits	Asparagine (mg/kg dough)	Asparagine reduction (%)
Control	122.7 ± 12^{a}	
1000 ASNU	40.2 ± 7.3^{b}	68
2000 ASNU	6.6 ± 1.8^{c}	95
3000 ASNU	1.5 ± 0.4^{c}	99
1000 ASNU + 15 Min RT	19.2 ± 1.6^{bc}	85
2000 ASNU + 15 Min RT	2.1 ± 0.3^{c}	98
3000 ASNU + 15 Min RT	0.2 ± 0.3^{c}	100
2000 ASNU + 30 Min RT	4.9 ± 0.3^{c}	96
2000 ASNU + 15 Min x 37 °C RT	1.1 ± 0.6^{c}	99
2000 ASNU + MS	10 ± 0.1^{c}	92
2000 ASNU + MT	7.3 ± 2.7^{c}	94
2000 ASNU+ MS x MT	5.6 ± 1.3^{c}	96
2000 ASNU + S	13.8 ± 2.5^{c}	89

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, S: Shortening, RT: Resting Time, MS: Mixing Speed, MT: Mixing Time.

Asparagine concentration and asparagine reduction of the sheeted dough samples was given in Table 4.2. In the literature the free asparagine content of wheat flour was reported to range from 163 to 664 mg/kg flour [81]. While the free asparagine content was at the level of 200 mg/kg flour in sifted flours, it was around 500 mg/kg flour in whole wheat flour [58]. Accordingly, the free asparagine concentration of the biscuit flour used in this study was below the reported levels in the literature. Lower levels of free asparagine in the flours could be considered as an advantage as lower levels of precursors mean lower levels of acrylamide depending on the processing conditions.

In 1000 ASNU treatment, asparagine was reduced by 68% in comparison to control. In 2000 ASNU treatment, asparagine reduction ratio increased to 95% and this increase was statistically significant (p<0.05) compared to 1000 ASNU treatment. In 3000 ASNU treatment, 99% asparagine reduction was obtained and there was 1.5 ± 0.4 mg asparagine/kg dough. However, asparagine reduction in 3000 ASNU treatment was found not to be significant (p>0.05) compared to 2000 ASNU treatment.

There was an increase in asparagine reduction (Table 4.2), although there was no significant difference between 1000 ASNU + 15 Min RT, 2000 ASNU + 15 Min RT, and 3000 ASNU + 15 Min RT treatments which had a resting period and only asparaginase enzyme applied doughs (1000, 2000, 3000 ASNU treatments), respectively (p>0.05). Although the difference in asparagine reduction between 3000 ASNU + 15 Min RT and 3000 ASNU treatments was low, difference in their acrylamide content and acrylamide reduction was higher indicating that even small amount of changes in asparagine content of dough may result in higher alteration in acrylamide formation of biscuits.

To observe the effect of increasing the resting time on the reduction of asparagine, 15 min and 30 min resting time was applied to dough at one dosage (2000 ASNU). The difference between the reduction in asparagine after 2000 ASNU + 15 Min RT (98%) and 2000 ASNU + 30 Min RT (96%) treatments was found to be not significant (p>0.05) (Table 1.2).

To understand the resting temperature on asparagine reduction, 2000 ASNU + 15 Min x 37 °C RT and 2000 ASNU + 15 Min at 25 °C treatments were applied to dough, respectively. No statistically significant difference was found in the asparagine concentration of the dough after 2000 ASNU + 15 Min x 37 °C RT (1.1 ± 0.6 mg asparagine/kg dough) and 2000 ASNU + 15 Min RT (2.1 ± 0.3 mg asparagine/kg dough) treatments (p>0.05). It showed that the temperature increase during the resting period did not have a significant effect on the activity of asparaginase in sheeted dough (p>0.05).

There was no statistically significant difference (p>0.05) between the asparagine reduction levels of 2000 ASNU + MS, 2000 ASNU + MT, 2000 ASNU + MS x MT applications and the 2000 ASNU (p>0.05). This indicated that mixing speed and time did not affect asparagine mitigation.

The difference between the asparagine reduction of 2000 ASNU + S (89 %) and 2000 ASNU (95%) is insignificant (p>0.05). This supported the fact that the fat did not have either a negative or a positive effect on acrylamide formation.

4.1.2. Effect of Asparaginase Treatments on Acrylamide Reduction in Rotary Dough Biscuits and Asparagine Reduction in Rotary Dough

To achieve acrylamide reduction in rotary dough biscuits, addition of resting time to the dough, changing resting temperature and time, adding sodium bicarbonate instead of ammonium bicarbonate, changing mixing order of the ingredients was applied.

Table 4.3. Acrylamide content (µg/kg sample) and acrylamide reduction (%) in rotary dough biscuit after asparaginase enzyme treatments.

Treatments in rotary dough biscuits	Acrylamide (µg/kg sample)	Acrylamide reduction (%)
Control	423 ± 71^{a}	
2000 ASNU	399 ± 22^{a}	6
2000 ASNU+ 15 Min RT	438 ± 9^{a}	-
2000 ASNU + 15 Min x 37 °C RT	496 ± 5^{a}	-
2000 ASNU + $NaHCO_3$	479 ± 132^{a}	-
2000 ASNU + MOC	412 ± 109^{a}	3
5000 ASNU+ 30 Min RT	500 ± 75^{a}	-

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time, MOC: Mixing Order Change.

In rotary dough biscuits, significant difference was not found in the acrylamide concentration of the control and after 2000 ASNU treatment to the dough (Table 4.3) (p>0.05). Additionally, there was no statistically significant difference between the control and the 5000 ASNU+ 30 Min RT treatment (p>0.05). It was revealed that the enzyme did not work optimally in this biscuit group, so a stepwise increase in the asparaginase dosage was not applied for rotary dough.

To reveal the effect of the resting period on acrylamide formation in rotary dough biscuits, application of 2000 ASNU treatment and 2000 ASNU+ 15 Min RT treatment to the rotary dough was compared, respectively. The acrylamide content of rotary dough biscuit after 2000 ASNU treatment was 438 ± 9 µg acrylamide /kg sample and it was 399 ± 22 µg acrylamide/kg sample after 2000 ASNU+ 15 Min RT treatment, respectively. Additionally, there was no significant difference in acrylamide concentrations of biscuits after these treatments (Table 4.3) (p>0.05). It was found that even resting period did not

affect the activity of the enzyme in the rotary dough matrix. Moreover, acrylamide content of biscuits after 5000 ASNU+ 30 Min RT application to rotary dough was not different from the control (p>0.05). Hence, resting time was not extended as the enzyme did not work even during 30 min resting time.

In rotary dough biscuits, the effect of resting temperature was examined. There was no significant difference in terms of acrylamide content of biscuits after 2000 ASNU+ 15 Min RT and 2000 ASNU + 15 Min x 37 °C RT treatment applied to dough (Table 4.3) (p>0.05). This result showed that the resting temperature has no effect on the activity of asparaginase in rotary dough since the enzyme cannot achieve optimum working conditions in this matrix.

The difference between the acrylamide content of the biscuits after 2000 ASNU + $NaHCO_3$ and the 2000 ASNU treatments of dough was not significant (p>0.05). Accordingly, subsequent addition of sodium bicarbonate to the matrix has no effect on acrylamide formation.

There was no significant difference between the reduction of acrylamide in biscuits after application of 2000 ASNU + MOC and 2000 ASNU treatments to dough (p>0.05). The purpose of the mixing change order was to increase the contact time by interacting the asparaginase enzyme with the flour before. Bringing the intended enzyme-substrate together earlier by changing the mixing order did not affect acrylamide formation.

Table 4.4. Asparagine concentration (mg/kg dough) and asparagine reduction (%) in rotary dough biscuit after asparaginase enzyme treatments.

Treatments in rotary dough biscuits	Asparagine (mg/kg dough)	Asparagine reduction (%)
Control	113.5 ± 2.3^{ab}	
2000 ASNU	113.3 ± 4.5^{ab}	-
2000 ASNU+ 15 Min RT	113.8 ± 2.1^{ab}	-
2000 ASNU + 15 Min x 37 °C RT	103 ± 13.2^{abc}	9
2000 ASNU + $NaHCO_3$	117.2 ± 5.5^{a}	-
2000 ASNU + MOC	87.6 ± 2.1^{c}	23
5000 ASNU+ 30 Min RT	90.4 ± 3.2^{bc}	20

*The same lowercase letters following the mean values indicate no statistical difference ($p<0.05$) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time, MOC: Mixing Order Change.

Rotary dough biscuits were examined in terms of their asparagine concentration. It was found that differences between the asparaginase applied to dough samples and the control was not significant (Table 4.4) ($p>0.05$). Additionally, there was no statistically significant difference between the asparagine concentration of the dough after 5000 ASNU+ 30 Min RT treatment and the control dough ($p>0.05$). No reduction in asparagine concentration even after high dosage asparagine addition and resting of dough could be regarded as the reason of no decrease in acrylamide formation in rotary dough biscuits.

To understand the effect of resting period in rotary dough biscuits, 2000 ASNU and 2000 ASNU+ 15 Min RT treatments were applied to the dough. It was found that there was no reduction in asparagine content of dough after 2000 ASNU+ 15 Min RT treatment compared to 2000 ASNU (Table 4.4) ($p>0.05$). Similarly, asparagine concentration of the dough after 2000 ASNU + 15 Min x 37 °C RT treatment was not different than asparagine content of the dough after 2000 ASNU+ 15 Min RT treatment ($p>0.05$) indicating that resting temperature did not affect asparagine reduction in rotary dough. In parallel to the acrylamide results, no decrease in the amount of asparagine was found after 2000 ASNU + $NaHCO_3$ treatment ($p>0.05$).

Contrary to the acrylamide results, a decrease (23%) was found in the asparagine concentration of the dough after 2000 ASNU + MOC treatment. Even this decrease was different from the 2000 ASNU treatment ($p<0.05$) there was no significant difference of acrylamide content these two applications.

4.1.3. Effect of Asparaginase Treatments on Acrylamide Reduction in Wire-Cut Biscuits and Asparagine Reduction in Wire-Cut Dough

Although there was no significant (p>0.05) change in acrylamide content of the wire-cut biscuits when the dough was treated with enzyme dosages up to 9000 ASNU/kg flour, the reduction was up to 30% (Table 4.5). There was a significant decrease in acrylamide content of biscuits when 12000 ASNU/kg enzyme dosage was applied (p<0.05). Additionally, in case of 12000 ASNU treatment, the acrylamide reduction was 37%.

Table 4.5. Acrylamide content (µg/kg sample) and acrylamide reduction (%) in wire-cut biscuit after asparaginase enzyme treatments.

Treatments in wire-cut biscuit	Acrylamide (µg/kg sample)	Acrylamide reduction (%)
Control	884 ± 118^{ab}	
1000 ASNU	872 ± 31^{ab}	1
2000 ASNU	935 ± 127^{a}	-
3000 ASNU	797 ± 26^{abc}	10
5000 ASNU	616 ± 104^{bcdef}	30
7000 ASNU	578 ± 36^{bcdef}	40
9000 ASNU	623 ± 73^{bcdef}	30
12000 ASNU	557 ± 11^{cdef}	37
1000 ASNU + 15 Min RT	862 ± 130^{ab}	2
2000 ASNU + 15 Min RT	774 ± 34^{abcd}	12
2000 ASNU + 30 Min RT	712 ± 0.1^{abcde}	19
3000 ASNU + 15 Min RT	657 ± 27^{bcdef}	26
5000 ASNU + 15 Min RT	542 ± 29^{cdef}	39
7000 ASNU + 15 Min RT	469 ± 38^{ef}	47
9000 ASNU +15 Min RT	480 ± 82^{ef}	46
9000 ASNU + 15 Min x 37 °C RT	443 ± 50^{ef}	50
12000 ASNU + 15 Min RT	505 ± 9^{def}	43
2000 ASNU + MOC	552 ± 29^{cdef}	38
5000 ASNU + MOC	406 ± 20^{f}	54
9000 ASNU + MOC	515 ± 8^{def}	42

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time, MOC: Mixing Order Change.

In a study conducted by Huang et al. [70], 0.5 U/g flour, 1 U/g flour, 2 U/g flour, 10 U/g flour, and 100 U/g flour asparaginase enzyme dosage was applied to short dough biscuit, respectively and as the enzyme dosage increased, more reduction in acrylamide formation was achieved until a certain enzyme dosage. While the highest acrylamide reduction was 90% at 10 U/g flour dosage, there was not significant difference between the 100 U/g flour and 10 U/g flour dosage flour application.

In wire-cut biscuit, there was no significant difference in any acrylamide reduction treatments that have resting time (15 min) at different dosages up to 12000 ASNU/kg flour and treatments with no resting time at the same dosages (p>0.05). This can be explained by the fact that the asparaginase enzyme started to work only at a dose of 12000 ASNU in the wire-cut biscuits as discussed above, but even at this dose, the resting period was not effective in this biscuit type, since the enzyme could not work effective enough. Acrylamide reduction in wire-cut biscuits after 2000 ASNU + 30 Min RT (19%) treatment to dough was not significantly different from 2000 ASNU + 15 Min RT (12%) (p>0.05). Because the enzyme could not work optimally at this dose, the increase in the resting time did not cause any decrease in acrylamide.

Since the enzyme started to show activity for the first time at 12000 ASNU in wire-cut biscuits, 9000 ASNU doses were chosen for the resting temperature increase to ensure early activity. The analysis performed showed no significant difference between the acrylamide content of the biscuits after 9000 ASNU +15 Min RT treatment to dough (69 ± 3 µg acrylamide/kg sample) and 9000 ASNU + 15 Min x 37 °C RT (57 ± 11 µg acrylamide/kg sample) (p>0.05). It was concluded that the increase in the resting temperature in the wire-cut dough matrix did not lead to a decrease in acrylamide.

To understand the effect of mixing order, the following treatments of 2000 ASNU + MOC, 5000 ASNU + MOC and 9000 ASNU + MOC were applied to wire-cut dough, respectively. Although acrylamide reduction of 38%, 54%, and 42% were achieved in wire-cut biscuits after 2000 ASNU + MOC, 5000 ASNU + MOC and 9000 ASNU + MOC treatments to dough, it was revealed that the acrylamide content of the biscuits after these applications was not different compared to the applications whose mixing order was not changed (p>0.05). Hence, it could be said that the changes in mixing order of the ingredients had no effect on acrylamide formation in wire-cut biscuits.

Table 4.6. Asparagine concentration (mg/kg dough) and asparagine reduction (%) in wire-cut biscuit after asparaginase enzyme treatments.

Treatments in wire-cut biscuit	Asparagine (mg/ kg dough)	Asparagine reduction (%)
Control	129 ± 10^{a}	
1000 ASNU	116 ± 1^{ab}	10
2000 ASNU	106 ± 4^{abc}	18
3000 ASNU	102 ± 7^{abcd}	21
5000 ASNU	101 ± 13^{abcd}	22
7000 ASNU	90 ± 4^{bcde}	30
9000 ASNU	87 ± 2^{bcde}	33
12000 ASNU	91 ± 14^{bcde}	30
1000 ASNU + 15 Min RT	117 ± 2^{ab}	9
2000 ASNU + 15 Min RT	102 ± 1^{abcd}	21
2000 ASNU + 30 Min RT	96 ± 1^{abcd}	26
3000 ASNU + 15 Min RT	104 ± 10^{abc}	19
5000 ASNU + 15 Min RT	79 ± 14^{cde}	39
7000 ASNU + 15 Min RT	73 ± 11^{cde}	43
9000 ASNU +15 Min RT	69 ± 3^{de}	47
9000 ASNU + 15 Min x 37 °C RT	57 ± 11^{e}	56
12000 ASNU + 15 Min RT	73 ± 9^{cde}	44
2000 ASNU + MOC	105 ± 1^{abc}	19
5000 ASNU + MOC	88 ± 12^{bcde}	32
9000 ASNU + MOC	98 ± 5^{abcd}	24

*The same lowercase letters following the mean values indicate no statistical difference ($p<0.05$) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time, MOC: Mixing Order Change.

Table 4.6 shows the changes in asparagine concentration of wire-cut dough after enzymatic processes at different dosages. There was no statistically significant difference between the asparagine concentration of enzyme dosages up to 9000 ASNU/kg flour and the control ($p>0.05$). After 7000 ASNU, 9000 ASNU and 12000 ASNU treatments, asparagine was decreased by 30%, 33% and 30%, respectively, and there was a significant change in asparagine concentration between these applications and the control ($p<0.05$). Therefore, asparaginase dosages higher than 7000 ASNU/kg flour did not effect mitigation of asparagine.

In wire-cut dough, the difference between the asparagine concentration the treatments with resting time (15 min) at different dosages up to 12000 ASNU/kg flour and the treatments with no resting time at the same dosages was not significant (p>0.05). It could be said that adding resting period did not have effect on reduction of asparagine.

In a study conducted by Alam et al. [82], 18 U/ml water of asparaginase was added onto model system composed of starch and asparagine and the model was rested for 30 and 60 min, and then baked. There was 90% of acrylamide reduction in the model rested for 60 min, while there was a 60% reduction in 30 min. The increase in the resting period increased the acrylamide concentration, but it should not be forgotten that this system was a model system and was free from the matrix effect.

The difference in the asparagine reduction of the dough after 2000 ASNU + 30 Min RT (26%) treatment and 2000 ASNU + 15 Min RT (21%) was not significant (p>0.05). Increasing resting time had no effect on the reduction of asparagine.

To understand the effect of resting temperature on the reduction of asparagine, the dough resting time was increased from 25 to 37 °C. Although more reduction was achieved 9000 ASNU + 15 Min x 37 °C RT (56%) compared to 9000 ASNU + 15 Min (47%), these two applications were not different from each other (p>0.05).

The asparagine concentration of the dough after 2000 ASNU + MOC, 5000 ASNU + MOC, and 9000 ASNU + MOC treatments were not different from those of 2000 ASNU, 5000 ASNU, and 9000 ASNU applications, revealing that changing mixing order had no effect on asparagine reduction.

4.1.4. Effect of Asparaginase Treatments on Acrylamide Reduction in Pretzel-like Snack and Asparagine Reduction in Pretzel-like Snack Dough

Effect of enzyme dosage, resting time and temperature on acrylamide reduction was investigated for pretzel-like snacks.

Table 4.7 Acrylamide content (µg/kg sample) and acrylamide reduction (%) pretzel-like snack after asparaginase enzyme treatments.

Treatment in pretzel-like snack	Acrylamide (µg/kg sample)	Acrylamide reduction (%)
Control	1377 ± 129^{a}	
2000 ASNU	757 ± 173^{b}	45
3000 ASNU	288 ± 76^{c}	79
2000 ASNU + 15 Min RT	458 ± 122^{bc}	67
3000 ASNU + 15 Min RT	279 ± 45^{c}	80
2000 ASNU + 15 Min x 37 °C RT	491 ± 72^{bc}	64

*The same lowercase letters following the mean values indicate no statistical difference ($p<0.05$) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time.

To reduce the formation of acrylamide in pretzel-like snack, 2000 ASNU and 3000 ASNU treatments were carried out and acrylamide contents were listed in the Table 4.7. In the treatment of 2000 ASNU, the formation of acrylamide was reduced by 45% and acrylamide content of the pretzel-like snack after this treatment was significantly less than the amount of acrylamide in the control ($p<0.05$). After 3000 ASNU treatment, 79% reduction was achieved in pretzel-like snacks. There was significant reduction in the acrylamide concentration of 3000 ASNU compared to the control ($p<0.05$). Acrylamide reduction in 3000 ASNU was higher than 2000 ASNU and there was a significant difference between these applications ($p<0.05$). By using 2000 ASNU/kg flour, it was not possible to lower the acrylamide level below 400 µg acrylamide/kg sample, which is the benchmark level determined for crackers. Further increase of the enzyme dosage to 3000 ASNU/kg flour achieved to reduce acrylamide below the benchmark level. Therefore, it was concluded that increasing the asparaginase dosage in pretzel-like snack dough had a positive effect on asparagine reduction in pretzel-like snack.

In a study published by Kumar et al. [83], different doses of asparaginase (50 U, 100 U, 200 U and 300 U) enzymes were applied to sweet breads and it was revealed that the amount of acrylamide formed after cooking decreased as the enzyme dosage increased. The highest percentage of acrylamide reduction in both the crust and crumb of the sweet bread was 97% and 73% when the highest dosage of 300 U applied, respectively.

There was no statistically significant difference in the asparagine reduction of the treatments with resting period (2000 ASNU + 15 Min RT (67%) and 3000 ASNU + 15 Min RT (80%)) and the treatments with no resting time (p>0.05). These results showed that the resting period in pretzel-like snacks did not have a positive effect on reducing acrylamide formation after asparaginase addition to dough. The reason for that may be the restriction of the enzyme activity due to higher pH resulted in the spraying of 1% NaOH solution on the pretzel-like snack before resting period. Resting of the pretzel-like snack sticks for 30 min could not be performed because it caused excessive drying due to water loss.

The acrylamide content of the pretzel-like snacks after 2000 ASNU + 15 Min treatment (458 ± 122 µg/kg sample) and 2000 ASNU + 15 Min x 37 °C RT treatment (491 ± 72 µg/kg sample) were not statistically different from each other (p>0.05). It was found that resting temperature had no effect on asparaginase activity in pretzel-like snack.

Table 4.8 Asparagine concentration (mg/kg dough) and asparagine reduction (%) in pretzel-like snack after asparaginase enzyme treatments.

Treatment in pretzel-like snack	Asparagine (mg/kg dough)	Asparagine reduction (%)
Control	126.1 ± 8.2[a]	
2000 ASNU	26.2 ± 11.9[b]	79
3000 ASNU	12 ± 5.8[b]	90
2000 ASNU + 15 Min RT	24.1 ± 0.5[b]	81
3000 ASNU + 15 Min RT	8.5 ± 1.5[b]	93
2000 ASNU + 15 Min x 37 °C RT	16.2 ± 6.2[b]	87

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time.

Compared to control, a decrease of 79% was found in the 2000 ASNU (p<0.05) (Table 4.8). Asparagine reduction increased to 90% after 3000 ASNU treatment compared to control but there was no statistically significant difference between 3000 ASNU and 2000 ASNU treatments (p>0.05).

81% and 93% reductions in asparagine concentrations of pretzel-like snack dough were achieved after 2000 ASNU + 15 Min RT and 3000 ASNU + 15 Min RT treatments, respectively. There was no significant difference between resting period applied treatments and the treatments with no resting period on the asparagine reduction (p>0.05).

In terms of asparagine content, there was no significant difference after 2000 ASNU + 15 Min x 37 °C RT (87%) and 2000 ASNU + 15 Min (81%) treatments to pretzel-like snack dough, respectively (p>0.05). These results showed that the increase in resting temperature of pretzel-like snacks did not cause a significant increase in asparaginase activity.

4.1.5. Effect of Asparaginase Treatments on Asparagine Reduction in Wafer Dough

Table 4.9. Asparagine concentration (mg/kg dough) and asparagine reduction (%) in wafer after asparaginase enzyme treatments.

Wafer	Asparagine (mg/kg dough)	Asparagine reduction (%)
Control	137 ± 5.9[a]	
1000 ASNU	4.7 ± 1.3[b]	97
2000 ASNU	6.4 ± 0.6[b]	95
1000 ASNU + 15 Min RT	6 ± 0.3[b]	96
2000 ASNU + 15 Min RT	5.6 ± 0.4[b]	96
2000 ASNU + 30 Min RT	5.6 ± 1[b]	96
1000 ASNU + 15 Min x 37 °C RT	4.8 ± 0.1[b]	96

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time.

There was no statistically significant difference in asparagine reduction in wafer dough after 2000 ASNU (95%) and 1000 ASNU (97%) treatments (p>0.05). This showed that the increase in dosage did not affect the reduction of asparagine because the enzyme

converted the asparagine in the dough to aspartic acid even at a dose of 1000 ASNU/kg flour.

There was no significant difference in the reduction of asparagine after 1000 ASNU + 15 Min RT and 2000 ASNU + 15 Min RT treatments compared to the treatments at the same dosages without a resting period (p>0.05). Hence, the resting period did not affect asparagine reduction as the enzyme already reduced asparagine at a high ratio without this period.

Asparagine reduction after 2000 ASNU + 15 Min RT treatment (96%) and 2000 ASNU + 30 Min RT treatment (96%) in wafer was not significantly different from each other (p>0.05). It could be suggested that extending the resting time in wafer dough did not have a positive effect on asparagine reduction.

There was not statistically significant difference in the asparagine concentration of the wafer dough after 1000 ASNU + 15 Min x 37 °C RT treatment (4.8 ± 0.1 mg/kg dough) and 1000 ASNU + 15 Min treatment (6 ± 0.3 mg/kg dough) (p>0.05). According to this result, increase in the resting temperature did not affect asparagine reduction in wafer dough.

4.2. Effect of Water Activity on Asparagine Content and Acrylamide Reduction in Bakery Products

Water activity levels of different dough types were given at Table 4.10. The highest water activity value was for wafer dough (0.97 ± 0.01). It was followed by the pretzel-like snack dough (0.92 ± 0.01) and sheeted dough (0.85 ± 0.02). The rotary dough had the lowest water activity (0.70 ± 0.01), but there was no statistically significant difference between the rotary dough and the wire-cut dough (0.74 ± 0.01).

Table 4.10. Water activity of different dough types.

Dough Type	Water activity
Sheeted dough	0.85 ± 0.02^{c}
Rotary dough	0.70 ± 0.01^{d}
Wire-cut	0.74 ± 0.01^{d}
Pretzel-like snack	0.92 ± 0.01^{b}
Wafer	0.97 ± 0.01^{a}

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test.

The asparaginase activity was the highest in wafer dough among all dough types. Even when 1000 ASNU/kg flour was applied, which was the lowest dosage used in all treatments, a 97% reduction in asparagine was successfully achieved. The main reason in this decrease was the highest water activity value (0.97 ± 0.01) and the optimum functioning of the enzyme.

Asparaginase was able to work effectively even at low dosages in pretzel-like snacks and sheeted dough biscuits because of their high-water activity values which were 0.92 ± 0.01 and 0.85 ± 0.02, respectively. Acrylamide content was reduced below benchmark levels and asparagine reduction rates of up to 96% was achieved in pretzel-like snacks and sheeted dough in parallel to their high-water activity values.

As the water activity decreases, the activity of the enzyme decreases. Accordingly, while a 65% reduction in acrylamide content was achieved when 2000 ASNU/kg flour dosage was used in sheeted dough biscuits, only 50% acrylamide reduction was achieved in wire-cut biscuits after 12000 ASNU/kg flour enzyme added to dough.

In rotary dough biscuits, whose dough has the lowest water activity, asparagine was reduced by 20% in some treatments, but significant acrylamide reductions were not achieved after most of the treatments. This revealed the effect of water activity on the activity of the asparaginase enzyme in sheeted dough and rotary dough, which has similar recipes except for their water compositions.

Hendriksen et al. [73], reported that asparagine enzyme was applied to gingerbreads with different moisture contents at a dosage of 1000 ASNU and a 90% reduction in acrylamide

formation occurred in the sample with the highest moisture content (19%), while a decrease of 19 % acrylamide reduction was obtained in the sample with a low moisture content (13%). This could be attributed to the loss of mobility of asparaginase and difficulty in contact of enzyme and substrate due to low amount of water.

4.3. Effect of pH on Asparagine Content and Acrylamide Reduction in Bakery Products

The pH values of the dough of the bakery products were presented for each treatment applied.

Table 4.11. pH values of sheeted dough samples.

Sheeted dough	pH
Control	8.4 ± 0.02^{c}
1000 ASNU	8.38 ± 0.04^{c}
2000 ASNU	8.41 ± 0.01^{c}
3000 ASNU	8.41 ± 0.01^{c}
2000 ASNU + S	8.44 ± 0.05^{bc}
1000 ASNU + 15 Min RT	8.4 ± 0.01^{c}
2000 ASNU + 15 Min RT	8.39 ± 0.01^{c}
3000 ASNU + 15 Min RT	8.41 ± 0.01^{c}
2000 ASNU + 15 Min x 37 °C RT	8.38 ± 0.03^{c}
2000 ASNU + MS	8.52 ± 0.02^{ab}
2000 ASNU + MT	8.51 ± 0.01^{ab}
2000 ASNU+ MS x MT	8.54 ± 0.01^{a}
2000 ASNU + 30 Min RT	8.44 ± 0.02^{bc}

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, S: Shortening, RT: Resting Time, MS: Mixing Speed, MT: Mixing Time.

The pH value of sheeted dough samples was given in Table 4.11. There were only small differences between applications in terms of pH value (p<0.05). The very limited pH variation between treatments showed that the reductions in acrylamide formation are independent of pH.

Table 4.12. pH values of rotary dough samples.

Rotary dough	pH
Control	7.55 ± 0.07^{b}
2000 ASNU	7.57 ± 0.04^{b}
2000 ASNU+ 15 Min RT	7.56 ± 0.01^{b}
2000 ASNU + 15 Min x 37 °C RT	7.69 ± 0.1^{b}
2000 ASNU + $NaHCO_3$	7.54 ± 0.08^{b}
2000 ASNU + MOC	8.01 ± 0.01^{a}
5000 ASNU+ 30 Min RT	7.57 ± 0.04^{b}

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time, MOC: Mixing Order Change.

The pH value of rotary dough samples was given in Table 4.12. Except for the pH value of the dough of 2000 ASNU + MOC treatment, significant differences were not found between the pH values of any application (p>0.05). The probable cause of the increasing of pH in 2000 ASNU + MOC was the delay in the pH decrease due to the slower coming into contact of the acid and the chemical leavening agents depending on the change in the mixing order.

Table 4.13. pH of wire-cut dough samples.

Wire-cut dough	pH
Control	7.47 ± 0.04^{b}
1000 ASNU	7.47 ± 0.03^{b}
2000 ASNU	7.57 ± 0.02^{b}
3000 ASNU	7.5 ± 0.07^{b}
5000 ASNU	7.58 ± 0.03^{b}
7000 ASNU	7.45 ± 0.07^{b}
9000 ASNU	7.54 ± 0.01^{b}
12000 ASNU	7.45 ± 0.07^{b}
1000 ASNU + 15 Min RT	8.06 ± 0.06^{a}
2000 ASNU + 15 Min RT	7.43 ± 0.04^{b}
2000 ASNU + 30 Min RT	7.48 ± 0.03^{b}
3000 ASNU + 15 Min RT	7.48 ± 0.01^{b}
5000 ASNU + 15 Min RT	7.48 ± 0.04^{b}
7000 ASNU + 15 Min RT	8.08 ± 0.04^{a}
9000 ASNU +15 Min RT	7.46 ± 0.01^{b}
9000 ASNU + 15 Min x 37 °C RT	7.45 ± 0.07^{b}
12000 ASNU + 15 Min RT	8.14 ± 0.08^{a}
2000 ASNU + MOC	7.48 ± 0.04^{b}
5000 ASNU + MOC	7.55 ± 0.04^{b}
9000 ASNU + MOC	7.55 ± 0.01^{b}

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time, MOC: Mixing Order Change.

The pH value of wire-cut dough samples was given in Table 4.13. It was found that there was not significant difference between the pH values of most applications (p>0.05).

Table 4.14. pH of pretzel-like snack dough samples.

Pretzel-like snack	pH
Control	7.91 ± 0.08^{a}
2000 ASNU	7.93 ± 0.01^{a}
3000 ASNU	7.89 ± 0.02^{a}
2000 ASNU + 15 Min RT	7.86 ± 0.06^{a}
3000 ASNU + 15 Min RT	7.98 ± 0.08^{a}
2000 ASNU + 15 Min x 37 °C RT	7.93 ± 0.01^{a}

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time.

The pH value of pretzel-like snack dough samples was given in Table 4.14. The pH values of any applications in pretzel-like snacks were not statistically different from each other (p>0.05).

Table 4.15. pH of wafer dough samples.

Wafer	pH
Control	6.99 ± 0.05[a]
1000 ASNU	7.02 ± 0.05[a]
2000 ASNU	7.01 ± 0.01[a]
1000 ASNU + 15 Min RT	7.02 ± 0.02[a]
2000 ASNU + 15 Min RT	7.01 ± 0.04[a]
2000 ASNU + 30 Min RT	6.99 ± 0.01[a]
1000 ASNU + 15 Min x 37 °C RT	6.98 ± 0.04[a]

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time.

The pH value of wafer dough samples was given in Table 4.15. As a result of the enzymatic processes applied to the wafer dough, it was found that the pH of any applications was not statistically different from each other (p>0.05).

Normally, while acrylamide formation is accelerated in alkaline conditions (pH 8) because of the amino group of asparagine protonated at alkaline pH is more likely to interact with the carbonyl group [52]. Also, the optimum pH of the enzyme from *Aspergillus oryzae* used in this study is 7 [69]. In practice, the pH of the matrices has moved away from the optimum pH (7) where the asparaginase enzyme will work, and has approached the pH (8) value where acrylamide formation is accelerated. In this case, it is expected that the decrease in asparagine will be limited and accordingly, there will be less decrease in the amount of acrylamide. However, contrary to expectations, pH differences in different product types did not affect acrylamide reduction.

Although the pH in pretzel-like snack and sheeted dough biscuits were higher than the pH of rotary dough biscuits and wire-cut biscuits, the reason for the high reduction of acrylamide reduction even in low asparaginase dosages could be related to that water activity is more effective on the activity of the asparaginase enzyme than pH in these bakery product types.

4.4. Interrelation Between Asparagine Content of Dough and Acrylamide Content of Bakery Products

The correlations between asparagine content of dough and acrylamide content of biscuits were evaluated as previously reported by Žilić et al. [84]. According to this study, correlations were as medium (R^2=0.6-0.7), high (R^2=0.7-0.9), very high (R^2>0.9) in different cereal flours [84].

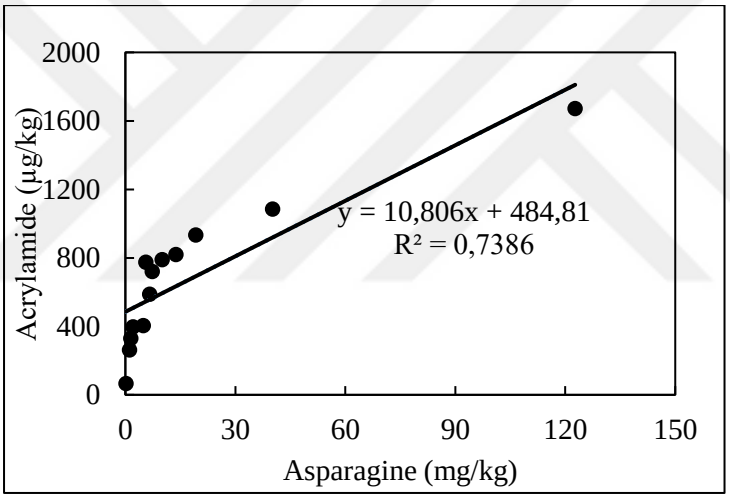


Figure 4.1. Interrelation between asparagine content of the sheeted dough (mg/kg) and acrylamide content of sheeted dough biscuits (µg/kg).

According to the results obtained in enzymatic applications in sheeted dough biscuits (Figure 4.1), the correlation coefficient (R^2) between asparagine and acrylamide was found to be 0.74. This value showed that there was a high positive correlation between these two independent variables (p<0.05).

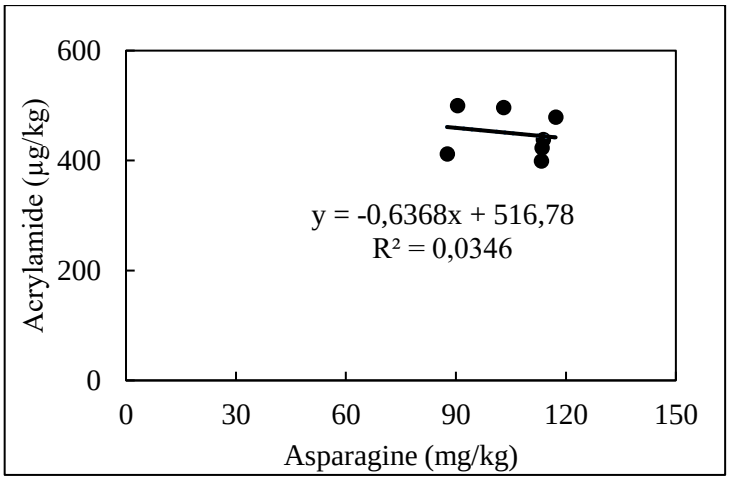


Figure 4.2 Interrelation between asparagine content of the rotary dough (mg/kg) and acrylamide content of rotary dough biscuits (µg/kg).

The correlation coefficient between the asparagine and acrylamide contents in the rotary dough biscuit was found to be 0.03 (Figure 4.2) (p<0.05). This showed that there was no relationship between these variables. As the asparaginase enzyme did not show activity in this biscuit type, sufficient reduction could not be achieved.

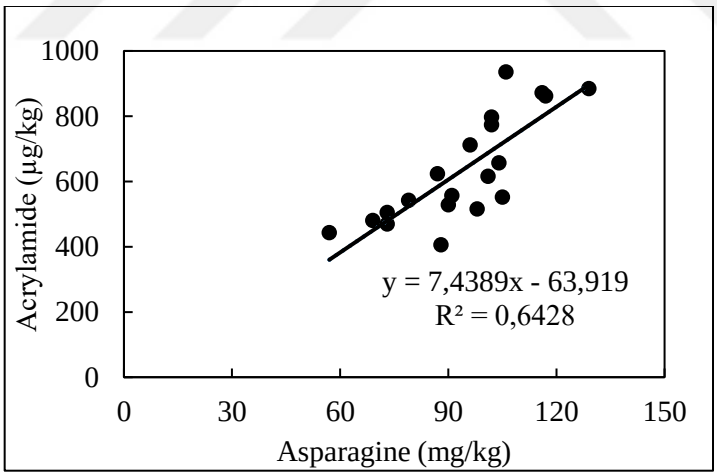


Figure 4.3 Interrelation between asparagine content of the wire-cut dough (mg/kg) and acrylamide content of wire-cut biscuits (µg/kg).

The correlation coefficient was calculated as 0.64 when asparagine contents and acrylamide contents were correlated in wire-cut biscuits (Figure 4.3) (p<0.05). This value revealed that there was a medium correlation between asparagine and acrylamide.

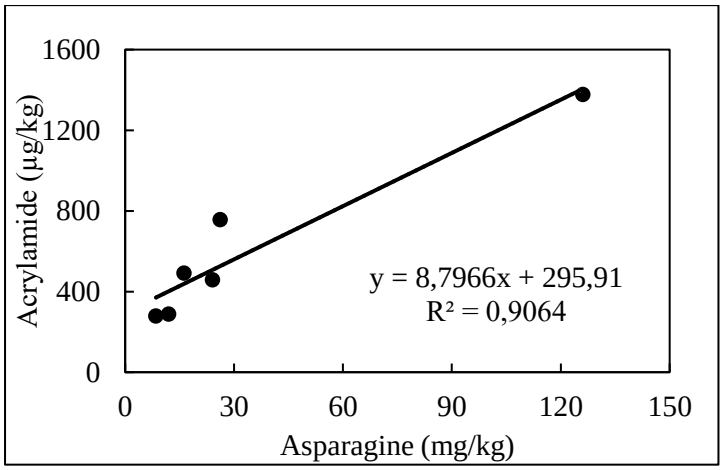


Figure 4.4 Interrelation between asparagine content of the pretzel-like snack dough (mg/kg) and acrylamide content of pretzel-like snack (µg/kg).

The correlation coefficient between asparagine contents and acrylamide contents in pretzel-like snack was found to be 0.91 (Figure 4.4) (p<0.05). This value revealed that there was a very high positive correlation between these two independent variables.

In general, there was a positive interrelation between asparagine and acrylamide. In wafer, only asparagine analysis was performed only, and a maximum 97% asparagine reduction was achieved. Considering the correlation coefficients of 0.74, 0.64 and 0.91 calculated in sheeted dough biscuits, wire-cut biscuits and pretzel-like snack, respectively, a high ratio of acrylamide reduction could be expected after baking the wafer dough when matrix effects are neglected. In parallel to this study, correlation studies conducted between the asparagine content of different flours and acrylamide in their biscuits revealed also medium, high, and very high correlation coefficients [84].

4.5. Effect of Acrylamide Reduction Strategies on Quality Parameters of Bakery Products

4.5.1 Spread Ratio

Spread ratio analysis was performed because of the physical properties of the product may change in case the dough dries out or gluten development occurs during the resting period applied to increase an enzyme activation.

Table 4.16. Spread ratio of sheeted dough biscuits.

Sheeted dough biscuits	Spread Ratio
Control	5.8 ± 0.06^{abcd}
1000 ASNU	5.7 ± 0.06^{d}
2000 ASNU	5.8 ± 0.04^{abcd}
3000 ASNU	5.7 ± 0.02^{bcd}
2000 ASNU + S	5.7 ± 0.03^{bcd}
1000 ASNU + 15 Min RT	5.7 ± 0.08^{cd}
2000 ASNU + 15 Min RT	5.8 ± 0.12^{abcd}
3000 ASNU + 15 Min RT	5.7 ± 0.08^{bcd}
2000 ASNU + 15 Min x 37 °C RT	5.7 ± 0.01^{cd}
2000 ASNU + MS	6.0 ± 0.06^{a}
2000 ASNU + MT	5.9 ± 0.01^{ab}
2000 ASNU+ MS x MT	5.8 ± 0.01^{abcd}
2000 ASNU + 30 Min RT	5.9 ± 0.01^{abc}

*The same lowercase letters following the mean values indicate no statistical difference ($p<0.05$) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, S: Shortening, RT: Resting Time, MS: Mixing Speed, MT: Mixing Time.

There was no significant difference between the spread ratio of any of the asparaginase enzyme treatments applied in the sheeted dough biscuits and the spread ratio of the control sample (Table 4.16) ($p>0.05$). It could be said that the asparaginase enzyme treatments had no effect on thickness or diameter of the sheeted dough biscuits and did not affect this quality parameter negatively.

Table 4.17. Spread ratio of rotary dough biscuits.

Rotary dough biscuits	Spread Ratio
Control	6.3 ± 0.04^{b}
2000 ASNU	6.6 ± 0.08^{ab}
2000 ASNU+ 15 Min RT	6.2 ± 0.07^{b}
2000 ASNU + 15 Min x 37 °C RT	6.1 ± 0.14^{b}
2000 ASNU + $NaHCO_3$	6.1 ± 0.02^{b}
2000 ASNU + MOC	7.2 ± 0.36^{a}
5000 ASNU+ 30 Min RT	6.4 ± 0.11^{b}

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time, MOC: Mixing Order Change.

Spread ratio of all treatments in rotary dough biscuit were the same as the control sample except for 2000 ASNU + MOC (Table 4.17) (p>0.05). The reason for the increase in the spread ratio after 2000 ASNU + MOC treatment to dough was the change in the mixing order. When the mixing order is changed, since the cream is added to the dough structure later, it was difficult for the water to bind to the matrix. Therefore, gluten did not easily reach water and gluten development may be decreased compared to other applications and the spread ratio may have increased. Additionally, the spread ratio was very close to each other in most applications with asparaginase enzyme and it did not affect the quality of rotary dough biscuits.

Table 4.18. Spread ratio of wire-cut biscuits.

Wire-cut biscuit	Spread Ratio
Control	7.7 ± 0.06^{abc}
1000 ASNU	7.6 ± 0.25^{abc}
2000 ASNU	7.5 ± 0.07^{abc}
3000 ASNU	7.4 ± 0.01^{bc}
5000 ASNU	7.4 ± 0.15^{c}
7000 ASNU	6.6 ± 0.1^{d}
9000 ASNU	7.7 ± 0.17^{ab}
12000 ASNU	7.7 ± 0.1^{abc}
1000 ASNU + 15 Min RT	7.5 ± 0.1^{abc}
2000 ASNU + 15 Min RT	7.6 ± 0.15^{abc}
2000 ASNU + 30 Min RT	6.7 ± 0.04^{d}
3000 ASNU + 15 Min RT	7.8 ± 0.02^{a}
5000 ASNU + 15 Min RT	7.7 ± 0.06^{abc}
7000 ASNU + 15 Min RT	7.7 ± 0.2^{abc}
9000 ASNU +15 Min RT	7.6 ± 0.07^{abc}
9000 ASNU + 15 Min x 37 °C RT	6.5 ± 0.02^{d}
12000 ASNU + 15 Min RT	7.6 ± 0.03^{abc}
2000 ASNU + MOC	7.6 ± 0.05^{abc}
5000 ASNU + MOC	7.6 ± 0.11^{abc}
9000 ASNU + MOC	7.5 ± 0.01^{abc}

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time, MOC: Mixing Order Change.

7000 ASNU, 2000 ASNU + 30 Min RT, 9000 ASNU + 15 Min x 37 °C RT applications in wire-cut biscuits had a lower spread ratio than the control (Table 4.18) (p<0.05). The reason for lower spread ratio in 2000 ASNU + 30 Min RT, 9000 ASNU + 15 Min x 37 °C RT treatments could be due to the increase in the resting time and the increase in biscuit thickness as a result of gluten development promoted by temperature. However, in general, in most of the treatments, no negative effect of the asparaginase enzyme on the spread ratio was observed in wire-cut biscuits.

Table 4.19. Spread ratio of pretzel-like snack.

Pretzel-like snack	Spread Ratio (Length/Diameter)
Control	36.0 ± 0.33^{a}
2000 ASNU	36.0 ± 0.20^{a}
3000 ASNU	36.1 ± 0.41^{a}
2000 ASNU + 15 Min RT	35.9 ± 0.36^{a}
3000 ASNU + 15 Min RT	35.9 ± 0.01^{a}
2000 ASNU + 15 Min x 37 °C RT	36.0 ± 0.19^{a}

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time.

There was not statistically significant difference between the spread ratio of asparaginase enzyme applied pretzel-like snack dough samples and the control (Table 4.19.) (p>0.05). Accordingly, it was concluded that the application of asparaginase did not have a negative effect on the spread ratio of pretzel-like snack.

In general, it could be said that the asparaginase enzyme applications did not affect the diameter and thickness values of biscuits in most of the samples which could be attributed the maintained rheological properties of dough. In the literature, there was no study reporting the effect of enzyme applications on the physical properties, which are height, diameter, or thickness, of biscuit types and pretzel-like snacks.

4.5.2 Color

Surface color values of bakery products were measured for each treatment and presented as L, a, b values.

Table 4.20. L, a, b color values of sheeted dough biscuit samples.

Sheeted dough biscuits	L	a	b
Control	71 ± 0.46^{a}	4.1 ± 0.24^{bc}	42 ± 0.77^{ab}
1000 ASNU	70 ± 0.72^{a}	4.2 ± 0.02^{bc}	41 ± 0.33^{ab}
2000 ASNU	69 ± 0.07^{ab}	4.3 ± 0.07^{bc}	42 ± 0.61^{ab}
3000 ASNU	70 ± 0.53^{a}	4.1 ± 0.19^{bc}	42 ± 0.49^{ab}
2000 ASNU + S	70 ± 0.84^{a}	3.7 ± 0.05^{c}	41 ± 0.78^{ab}
1000 ASNU + 15 Min RT	71 ± 0.39^{a}	4.2 ± 0.13^{bc}	42 ± 0.96^{ab}
2000 ASNU + 15 Min RT	71 ± 0.67^{a}	4.4 ± 0.1^{bc}	42 ± 0.79^{ab}
3000 ASNU + 15 Min RT	70 ± 0.38^{a}	4.4 ± 0.04^{bc}	42 ± 0.27^{ab}
2000 ASNU + 15 Min x 37 °C RT	68 ± 0.25^{b}	5.7 ± 0.9^{a}	41 ± 0.07^{b}
2000 ASNU + MS	71 ± 0.93^{a}	4.2 ± 0.07^{bc}	43 ± 0.17^{a}
2000 ASNU + MT	71 ± 0.52^{a}	4.5 ± 0.14^{bc}	43 ± 0.42^{ab}
2000 ASNU+ MS x MT	69 ± 0.06^{ab}	4.6 ± 0.22^{bc}	43 ± 0.24^{a}
2000 ASNU + 30 Min RT	70 ± 0.08^{a}	5.0 ± 0.03^{ab}	42 ± 1.0^{ab}

*The same lowercase letters following the mean values indicate no statistical difference ($p<0.05$) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, S: Shortening, RT: Resting Time, MS: Mixing Speed, MT: Mixing Time.

The L (brightness) values in the sheeted dough biscuits treated with asparaginase were listed in Table 4.20. Except for 2000 ASNU + 15 Min x 37 °C RT application, there was no statistically significant difference between the L values of all enzymatic processes and control sample ($p>0.05$). The reason why the L value of 2000 ASNU + 15 Min x 37 °C RT application (68 ± 0.25) was lower than L value of control may be the resting at 37 °C. Because as the initial dough temperature was slightly higher than the other applications, the dough reached to baking temperature more quickly and the surface of the biscuit became darker.

The b values given in the Table 4.20 represent the yellowness values for the sheeted dough biscuits. In terms of yellow color, there was not statistically significant difference in any of the asparaginase applications and the control (p>0.05). Applications in sheeted dough did not have a significant effect on the a value of the biscuits. (p>0.05). The small differences between the L, a, and b values in the applications showed that the asparaginase enzyme applications had no effect on the surface color of the sheeted dough biscuits.

Table 4.21. L, a, b color values of rotary dough biscuit samples.

Rotary dough biscuits	L	a	b
Control	65 ± 0.02^{ab}	3.5 ± 0.39^{bc}	39 ± 0.33^{a}
2000 ASNU	65 ± 0.21^{ab}	4.1 ± 0.25^{abc}	39 ± 0.24^{a}
2000 ASNU+ 15 Min RT	64 ± 0.09^{ab}	4.5 ± 0.6^{abc}	39 ± 1.21^{a}
2000 ASNU + 15 Min x 37 °C RT	61 ± 0.03^{b}	6.0 ± 0.46^{a}	40 ± 0.41^{a}
2000 ASNU + $NaHCO_3$	67 ± 2.99^{a}	2.4 ± 0.99^{c}	37 ± 1.13^{a}
2000 ASNU + MOC	64 ± 0.98^{ab}	4.6 ± 0.23^{ab}	37 ± 0.25^{a}
5000 ASNU+ 30 Min RT	65 ± 0.09^{ab}	3.6 ± 0.35^{bc}	39 ± 0.04^{a}

*The same lowercase letters following the mean values indicate no statistical difference (p<0.05) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time, MOC: Mixing Order Change.

There was not difference in the L and b values of rotary dough biscuits (Table 4.21) compared to control (p>0.05). Except for 2000 ASNU + 15 Min x 37 °C RT application, asparaginase enzyme treatment has no significant effect on the a value of rotary dough biscuits ($p>0.05$). This showed that the application of asparaginase enzyme to the dough had no effect on the quality of surface color in rotary dough biscuits.

Table 4.22. L, a, b color values of wire-cut biscuit samples.

Wire-cut biscuit	L	a	b
Control	70 ± 1.94^{bcde}	5.0 ± 0.81^{ab}	41 ± 1.09^{abcd}
1000 ASNU	69 ± 1.21^{bcde}	4.4 ± 0.13^{bc}	42 ± 0.28^{abc}
2000 ASNU	69 ± 1.02^{bcde}	5.0 ± 0.28^{ab}	43 ± 0.63^{ab}
3000 ASNU	70 ± 0.6^{bcde}	4.8 ± 0.64^{ab}	43 ± 0.51^{ab}
5000 ASNU	70 ± 0.27^{bcde}	5.1 ± 0.18^{ab}	41 ± 0.24^{abc}
7000 ASNU	68 ± 0.46^{cde}	5.1 ± 0.44^{ab}	43 ± 0.15^{ab}
9000 ASNU	69 ± 0.5^{bcde}	4.5 ± 0.88^{ab}	43 ± 0.81^{ab}
12000 ASNU	68 ± 0.64^{cde}	4.5 ± 0.15^{ab}	44 ± 0.89^{a}
1000 ASNU + 15 Min RT	74 ± 2.78^{ab}	5.2 ± 0.72^{cd}	39 ± 0.4^{cd}
2000 ASNU + 15 Min RT	70 ± 0.48^{bcde}	5.3 ± 0.05^{ab}	41 ± 0.23^{abcd}
2000 ASNU + 30 Min RT	68 ± 1.77^{cde}	4.8 ± 0.74^{ab}	44 ± 0.67^{a}
3000 ASNU + 15 Min RT	67 ± 2.97^{cde}	5.5 ± 1.62^{ab}	45 ± 1.12^{a}
5000 ASNU + 15 Min RT	70 ± 0.12^{bcde}	5.8 ± 0.24^{ab}	43 ± 0.17^{ab}
7000 ASNU + 15 Min RT	72 ± 0.78^{abc}	6.1 ± 0.15^{bcd}	42 ± 1.48^{abc}
9000 ASNU +15 Min RT	69 ± 0.19^{bcde}	4.1 ± 0.18^{b}	41 ± 0.38^{abc}
9000 ASNU + 15 Min x 37 °C RT	71 ± 0.45^{abcde}	7.5 ± 0.46^{bcd}	42 ± 0.07^{abc}
12000 ASNU + 15 Min RT	76 ± 1.88^{a}	7.3 ± 1.21^{d}	37 ± 0.43^{d}
2000 ASNU + MOC	72 ± 0.46^{abcd}	5.1 ± 0.21^{b}	40 ± 0.21^{bcd}
5000 ASNU + MOC	66 ± 0.04^{e}	7.2 ± 0.69^{a}	43 ± 0.69^{ab}
9000 ASNU + MOC	66 ± 0.19^{de}	8.1 ± 0.97^{a}	44 ± 1.09^{a}

*The same lowercase letters following the mean values indicate no statistical difference ($p<0.05$) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time, MOC: Mixing Order Change.

Significant differences between the L values of asparaginase enzyme applications and the control in the wire-cut biscuits ($p<0.05$), except the 12000 ASNU + 15 Min RT (Table 4.22). However, there was no difference in b values of any of the applications and the control ($p>0.05$). The a values of the wire-cut biscuits did not change significantly in most applications compared to control except for 1000 ASNU + 15 Min RT and 12000 ASNU + 15 Min RT ($p>0.05$). Accordingly, it was revealed that the application of asparaginase enzyme did not negatively affect the surface color quality of wire-cut biscuits.

Table 4.23. L, a, b color values of pretzel-like snack samples.

Pretzel-like snack	L	a	b
Control	71 ± 1.65[a]	1.3 ± 0.61[a]	32 ± 2.76[a]
2000 ASNU	69 ± 1.89[a]	1.8 ± 0.54[a]	30 ± 0.37[a]
3000 ASNU	69 ± 2.2[a]	1.1 ± 0.75[a]	33 ± 1.81[a]
2000 ASNU + 15 Min RT	69 ± 1.47[a]	0.5 ± 0.01[a]	34 ± 1.05[a]
3000 ASNU + 15 Min RT	71 ± 0.81[a]	0.5 ± 0.13[a]	32 ± 1.08[a]
2000 ASNU + 15 Min x 37 °C RT	66 ± 1.19[a]	0.7 ± 0.03[a]	35 ± 0.51[a]

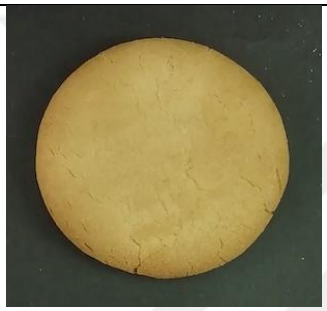
*The same lowercase letters following the mean values indicate no statistical difference ($p<0.05$) according to Tukey's test. ASNU: Amount of L-asparaginase that syntheses one micromole of ammonia per minute under standard conditions per kg flour, RT: Resting Time.

There was no difference between the enzymatic processes applied pretzel-like snacks and the pretzel-like snacks in the L, a, and b values compared to control ($p>0.05$) (Table 4.23). It could be said that the application of asparaginase enzyme did not affect the surface color of pretzel-like snacks.

There is no significant difference in surface color between the most effective enzyme treatments and control samples (Table 4.24). Studies in the literature support that the above-mentioned asparaginase enzyme does not have a negative effect on the surface color of bakery products. In a study conducted by Anese et al. [63], it was revealed that there was no statistically significant difference in color of 900 U/kg asparaginase enzyme added sample and the sample without asparaginase enzyme in short dough biscuits.

In a study conducted by Kukurova et al. [80], asparaginase enzyme was applied to cookie dough at a dose of 500 U/kg flour and the dough rested for resting times of 15 min, 30 min, and 60 min. As a result of baking, the L, a, b values of the cookies whose dough was enzymatically treated, and the control were not different significantly ($p>0.05$).

Table 4.24. Control and most effective enzyme treatment photograph of bakery products.

Control photograph of biscuit types	Most effective enzyme treatment of biscuits
 Control of sheeted dough biscuit	 3000 ASNU + 15 Min RT
 Control of rotary dough biscuit	 2000 ASNU
 Control of wire-cut biscuit	 5000 ASNU + MOC

5. CONCLUSION

To reduce acrylamide in different types of bakery products, enzymatic applications were carried out using the asparaginase enzyme. During these applications, the process conditions affecting the activity of the asparaginase enzyme were examined in different biscuit types (sheeted dough, wire-cut, and rotary dough) and pretzel-like snacks, and the optimum process conditions for the enzyme were determined. Enzyme dosage, resting period, resting time, resting temperature, adding shortening to the matrix afterwards, dough mixing speed, dough mixing time were the parameters tested for the activity of the asparaginase depending on the bakery product. Water activity and pH were the parameters affected by the composition of the bakery product and water activity was found to be more effective compared to pH.

Enzyme dosage, resting period, resting time, resting temperature, adding shortening to the matrix afterwards, and changing the dough mixing speed and time were investigated in sheeted dough biscuits that were baked at 230 °C for 8.5 min. It was determined that increasing enzyme dosage in the dough and adding dough resting period were quite effective on acrylamide reduction of sheeted dough biscuits. Addition of 3000 ASNU/kg flour to dough and 15 min dough resting time subsequently was the most effective treatment with 96% acrylamide and 100% asparagine reduction. By this application, acrylamide in sheeted dough biscuits was reduced below the benchmark level of biscuits. Additionally, this application had no adverse effect on surface color and spread ratio of sheeted dough biscuits.

As the water activity of rotary dough biscuits baked at 210 °C for 8.5 is quite low (0.70), asparaginase could not be fully activated. Therefore, even if a limited conversion from asparagine to aspartic acid occurred, no significant decrease was found in acrylamide formation. It could be possible to stress that water activity was so important that it even did not allow to test the other parameters by preventing the function of enzyme. The reason for the remained surface color and spread ratio of rotary dough biscuits was again the inability of enzyme to perform any activity in rotary dough biscuits.

To examine the factors affecting the activity of asparaginase in wire-cut biscuits baked at 210 °C for 8 min; enzyme dosage, resting period, resting time, resting temperature, and changing the mixing order of ingredients were applied to dough. As a result of these applications, it was concluded that increasing the enzyme dosage was the only effective treatment, but the significant reduction in acrylamide only occurred when 12000 ASNU/kg flour dosage applied to wire-cut dough. The most effective acrylamide reduction (54%) was achieved when 5000 ASNU/kg flour dosage added to dough together with changing of mixing order. Due to the low amount of water activity (0.74) in wire-cut dough compared to sheeted dough, the enzyme asparaginase was able to reduce acrylamide to a certain level. Additionally, enzyme treatments did not have any negative effect on the surface color or spread ratio of wire-cut biscuits.

Enzyme dosage, resting period, resting time, and resting temperature were the parameters applied to pretzel-like snack dough which was baked at 210 °C for 9 min. It was found that the increasing enzyme dosage from 2000 ASNU/kg flour to 3000 ASNU/kg flour and the resting period from 15 min at lower enzyme dosages were effective in the reduction of acrylamide. Maximum acrylamide reduction (%80) was achieved by addition of 3000 ASNU/kg flour to dough and subsequent resting for 15 min and the acrylamide benchmark level determined for pretzel-like snacks was successfully achieved. It was revealed that asparaginase enzyme applications did not have any negative effect on the quality parameters examined in pretzel-like snacks.

The correlation coefficients between the amount of acrylamide in bakery products and the amount of free asparagine in their dough revealed that there was a positive correlation between asparagine and acrylamide in all products (sheeted dough biscuits r: 0.74, wire-cut biscuits r: 0.64, pretzel-like snacks r: 0.91) except for the rotary dough where the enzyme was not active. Accordingly, as asparagine was reduced by 97% in wafer dough, it could be expected in wafers a high percentage of acrylamide reduction.

It could be concluded that the results obtained within the scope of this thesis will contribute both to the literature and the food industry. The acrylamide reduction strategies evaluated for each bakery product will guide the industry to reduce acrylamide below benchmark levels which could be necessary to prevent regulation related problems during export and to make industry be prepared for the upcoming regulations.

In the light of all these, the importance of determination of the pH and water activity of the dough in different bakery products before the treatment of the asparaginase enzyme was stressed. Moreover, addition of a resting period, prolonging resting time, and increasing the dough temperature by themselves or in combinations were suggested to be applied to bakery products by considering the activity of enzyme.

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