ACRYLAMIDE REDUCTION OPTIONS IN RYE BREAD

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Abstract

Acrylamide is a food contaminant can be formed in foods when heated above 120 °C, if carbohydrates, especially reducing sugars, and asparagine are present. Bread is among the products that can contain high levels of acrylamide. The aim of this study is to evaluate acrylamide content in bread samples from different varieties of rye grains from different soils and to analyse acrylamide content reduction possibilities. Within the framework of the research, soil sulphur sufficiency in experimental fields of rye was analysed, and the content of amino acids was determined in rye grains, dough and bread by using standard methods. The samples were obtained from rye grain, flour and bread, then the impact of enzyme asparaginase, rosemary extract, and citric acid on the acrylamide content in the bread were analysed. The rye bread baking tests were carried out in an industrial bakery. The results of the soil analysis indicated a deficiency of sulphur containing compounds. The usage of enzyme asparaginase in rye bread production caused no significant reduction ($p>0.05$) of acrylamide content in rye bread. Analysis of the economic aspects of asparaginase utilization concluded that its utilization in production is not cost effective. The addition of rosemary extract to rye dough did not reduce the acrylamide content in bread. The addition of citric acid to rye dough reduced the content of acrylamide in rye bread by 66%, but had a negative effect on taste. Therefore, it is necessary to find the optimum quantity of citric acid that can be added to bread without changing the sensory properties of acidity and flavour.

Keywords: rye bread, acrylamide, asparaginase, citric acid, rosemary extract.

Introduction

Swedish scientists found acrylamide in food in 2002 (Yuan, 2011; Claus et al., 2008a). At present, a lot of research has been carried out in the world to understand its formation mechanism and to reduce the content of acrylamide in foodstuffs. Investigations made by Maastricht university (Hogervorst et al., 2007), indicate close correlation between acrylamide quantity in food and cause of separate cancerous disease. The International Agency for Research on Cancer is classified acrylamide as probably carcinogenic (2A group) to humans (IARC, 1994), however indistinctness still exist in that matter, because several investigations deny it (Laroque et al., 2008).

Acrylamide is substance, which may be form in starch containing products processed in high temperature (above 120 °C) – by fritting, frying, toasting. The most concentration of acrylamide is in bread`s crust (Brathen, Knutsen, 2005).

Several scientists concluded that acrylamide forms during Maillard reaction (Zyzak et al., 2003; Stadler et al., 2002; Becalski et al., 2003). Investigations showed that acrylamide does not form in boiled starch containing products. It is possible decomposing of acrylamide at protractedly frying at high (200 °C) temperature (Rydberg at al., 2003, Ahrne at al., 2007).

Factors what promote formation of acrylamide in foodstuffs are different, and strengthening each other. The most important of these is presence of amino acid asparagine, reducing sugar fructose as well as temperature, treatment time, water activity. Asparagine (Asn) is neutral amino acid and results of scientific investigations show that it is one of the main causes of acrylamide in bread and bakery products (Zhang et al., 2009).

Asparagine is soluble in alkalis, acids, and forms white monohydrate crystals with water.

The ratio of N : C in asparagin molecule is 2 : 4 which provide reserve and transportation of nitrogen in plants (Lea, 2007). Asparagine accumulates in plants under stress conditions. The growth of asparagine by 50% in wheat is observed under sulphur deficiency conditions. Cereals have been little studied in this area but existing investigations show that genetic as well as environmental factors dramatically affect asparagine in cereals (Zhang et al., 2009, Halford et al., 2007). Latvia no data about asparagine content in grain products, up to now in amino acids analysis was determined acidic amino acid - aspartic acid (Asp).

Swedish researchers found that the addition of asparagine dough stated notable acrylamide growth in bread from 80 to 6000 ppm, 99% of this amount was in crust (Yaylayan et al., 2003; Mottram et al., 2002).

European Food Safety Authority (EFSA) Scientific Panel on 19 April 2005 adopted statement on acrylamide in food where confirmed the need for the risk assessment. The EU has not imposed restrictions on the acrylamide concentration in food but has developed recommendations for its reduction, as well as EFSA incorporated data from manufacturers about its content in food. EFSA acrylamide indicative figures mentioned that fresh bread should not exceed 150 $\mu g\ kg^{-1}$, crisp-bread – 500 $\mu g\ kg^{-1}$ of acrylamide (EFSA, 2009).

The Scientific literature (Claus et al., 2008a) data on acrylamide content in bread and bakery products has wide range due not only to different bread recipes and dough making technology but also to the kind of oven used and the kind of baking process.

Agronomical measures are recommended carry out in order to reduce acrylamide content in bread as well as make changes in the recipe and technology. Scientists recommended controlling sulphur level in the soil during cereals breeding as agronomical measure. This is reflected in the study where sulphate deficiency in

cereals during breeding period called dramatic increases in the concentration of asparagine in grains and unexpectedly high acrylamide content in bread (Muttucumaru et al., 2006; Claus et al., 2008b).
German scientists investigating asparagine in rye have found that it is 319–791 mg kg^{-1} (Springer et al., 2003). There no investigations in Latvia about asparagine content in rye and bread but there are data about aspartic acid content in grain samples of five rye varieties as 0.55–0.70 g 100 g^{-1} (Straumite, 2006, Ozolina, 2012).
Following the guidelines of CIAA, would be useful to develop cooperation between farmers, manufacturers and scientists on sulphur containing fertilizers utilization on cereals cultivation, as well as on new cereals varieties containing as low as possible amount of asparagines (CIAA, 2009).
The aim of study is to evaluate acrylamide content in bread samples from different varieties of rye grains and to analyse acrylamide content reduction possibilities.
Within the framework of the research, soil sulphur sufficiency in experimental fields of rye was analysed, and the content of amino acids was determined in rye grains, dough and bread. The samples were obtained from rye bread baking tests, then the impact of enzyme asparaginase, rosemary extract, and citric acid on the acrylamide content in the bread were analysed. The rye bread baking tests were carried out in an industrial bakery. The content of amino acids in dough and bread was determined, and aspartic acid relevance to acrylamide formation was evaluated.

Materials and Methods

The Influence of Meteorogical Conditions
Winter rye was sown in late September in weather conditions favourable to winter crops.
Snow cover formed in the last ten days of October yet did not cause significant damage and winter rye growth continued until the second ten days of November. At the end of the active growth period, winter crops had begun their tillering phase. In total, the winter months were colder and richer in precipitation compared with long -term mean data.
As the ground under the snow was not frozen, it delayed the plants overheating under a thick snow blanket ensuring their survival. In the spring of 2013 winter receded slowly, in the first ten days of April the average temperature was negative, the winter crops recrudesced in the second ten day period of April which is about a week later than usual. April was cool, the warm weather arrived in the first ten days of May, the second and third ten-day periods of May were rainy. The summer of 2013 was warmer and drier than usual. Consequently, winter crop development was faster; they even ripened earlier than usual, at the end of July. Due to this rapid development yield was lower than in 2012.

Materials and methods used in bread baking tests
A hybrid variety of rye ‘Agronom’ and population variety of rye ‘Kaupo’ cultivated at the State Plant Breeding Institute Priekuli and State Cereals Breeding Institute Stende trial fields, were used in the research. Cleaned and prepared for processing, the grain samples were ground in a *Hawos Queen2* mill at the Bread Manufacturing Laboratory of Latvia University of Agriculture, Faculty of Food Technology and whole meal rye flour with moisture content of 11.2 to 12% was obtained for bread baking
The enzyme asparaginase (*Megazyme)* with activity: 15 U/mg (25 °C, pH 8.0), the natural antioxidant rosemary extract „Oleoresin Rosemary 41-19-25” (recommended amount of addition 0.08% to dough mass), and citric acid (E330) were used in the baking tests.

Whole meal rye bread bakery tests
Bread bakery tests were carried out to assess enzyme asparaginase, rosemary extract and citric acid impact on acrylamide formation intensity in rye bread samples, which were prepared in an industrial module system.
Hybrid variety ‘Agronom’ and population variety ‘Kaupo’ whole meal rye flour, sugar, salt and water were used for the dough preparation. The dough recipe is shown in Table 1. The dough was prepared in accordance with bread making technology, including starter preparation, dough kneading and fermentation, dough dividing, loaf formation and baking.

Table 1

Whole meal rye bread basic recipe

Semi-finished product	Raw materials	Amount of product, g
Yeast bread	Rye whole meal flour	250
	Water	250
Dough	Yeast bread	500
	Rye whole meal	250
	Salt	10
	Sugar	30
	Water	150
Total		940

The starter was made using German *Böcker* pure rye bread starter culture. The starter was prepared separately for each flour sample, the necessary temperature (28–30 °C) and fermentation time (2×24 hours) were provided. 50% of the total quantity of rye flour was used for the starter. The starter acidity ranged from pH 4.0 to 3.9, which corresponds to the required acidity for rye bread starter.
The dough was mixed for 10 minutes until all the ingredients were evenly blended and a homogeneous mass resulted. Enzyme asparaginase (500 U) was added to four samples at the end of dough mixing. The dough fermented for 30 minutes, then was divided into 400 gram loaves, then put into forms and fermented for 60 minutes in the post-fermentation chamber at a temperature of 35 °C and 70% relative air humidity.

Fermented semi-finished dough was baked in a *Sweba Dahlen* convection oven at a temperature of 200 °C for 40 minutes. After baking, the bread samples were cooled and their taste was evaluated. The bread samples were baked for 50 minutes in the clay oven at a falling temperature regime (initial temperature 260 °C, final temperature 210 °C). The added rosemary extract Oleoresin Rosemary 41-19-25 proportion was 0.1% and 0.5%, and citric acid proportion was 1%, following the manufacturer's recommendations. The baking tests for rye bread with dried fruits, with and without the addition of rosemary extract (0. 1%), were conducted at the bakery.

Amino acids in flour, dough and rye bread samples
Amino acids were analysed according to AOAC standard method N. 994.12, by using ion change after sample hydrolysis by 6M HCl in an inert atmosphere. The content of amino acids was determined by using an Automatic Amino Acids Analyzer (Microtechna Praha), and as the standard, the „Amino acid Standard solution for protein hydrolysates 0.5 µmoles per mL" (Sigma).

Acrylamide Acrylamide separation from the bread samples was achieved with solid phase extraction, but the acrylamide content was analysed with liquid chromatograph-tandem mass spectrometry (LC-MS/MS). For analysis 2±0.0001 g of dried and homogeneous sample was weighed into a 50 mL centrifuge tube. The internal standard was added (d3-acrylamide) at 100 µg kg^{-1}. Then 5 mL of hexane was added for sample degreasing, and then the tube was vortexed. After 10 mL of water, 10 mL of acetonitrile, 4.0 g $MgSO_4$ and 0.5 g NaCl were added the tube was vigorously shaken for 1 min, then centrifuged at 4000 rpm for 5 min. The hexane layer was discarded and 1 mL of the acetonitrile extract was transferred to a tube containing 50 mg of primary and secondary amines (PSA) and 150 mg $MgSO_4$. The tube was vortexed for 30 sec and then placed in an autosampler vial, and analysed with LC-MS/MS (Waters 2695, Quattro Premire XE). The determination of acrylamide was performed at the Latvia Institute of Food Safety, Animal Health and Environment "BIOR"

Statistical analysis. Experimental data evaluation was done using two–factor analysis of variance by Fisher's criteria and least significant difference ($LSD_{0.05}$). Components of variance ANOVA for each quality characteristic were expressed as percentages to illustrate the relative impact of each source to the total variance.

Results and Discussion

Field trials and rye cultivation
Hybrid rye variety 'Agronom' and population rye variety 'Kaupo' were grown in field trials. Scientific literature indicates that rye hybrid varieties generally are higher yield than rye population varieties (Petr, 2006). In this 2013 trial, such a difference was not observed. This may be due to the relatively rapid development of the plants in the summer of 2013, as a result hybrid rye have not realizing its potential for yield. The 2013 yield was influenced significantly by fungicide usage at sowing – both varieties have significantly higher yield with fungicide usage – more than one tonne per hectare (Table 2).

Table 2

Yield of hybrid and population rye varieties, t h^{-1}

Variety	NPK+R+H	NPK+R+H+F	Average
'Agronom' F1 (hybrid)	5.62	6.65	6.13
'Kaupo' (population)	5.73	6.48	6.11

In 2013 there was no significant difference between the tested populations of hybrid varieties of rye and yields. The yield of both varieties of rye in the presence of fungicide increased. Samples were submitted from both varieties harvested and submitted for further analysis. The study locations were the State Plant Breeding Institute Priekuli (hereinafter referred to as Priekuli) and State Cereals Breeding Institute Stende (hereinafter referred to as Stende).

Table 3

The soil agrochemical parameters

Sample of soil	Reac-tion	Organic matter	P_2O_5	K_2O	$S\text{-}SO_4$
	pH, HCl	%	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Stende 1	5.4	1.7	63.0	121.0	4.0
Stende 2	4.0	4.0	271.0	145.0	1.2
Stende 3	4.0	2.1	256.0	140.0	1.2
Priekuli 1	4.8	1.9	95.0	106.0	0.6
Priekuli 2	6.0	2.5	101.0	88.0	0.6
Priekuli 3	4.9	2.1	100.0	127.0	0.6

In both study sites the research was carried out on the soil of relatively poor sod-podzoloic and sod-podzoloic sandy loam. The agro-chemical characteristics of the soil were assessed in accordance with the March 15th, 2007, Ministry of Agriculture (Latvia's) guideline No. 12 "Methodological procedures for agro-chemical soil exploration and evaluation of the results", as outlined in Table 3. Stendes soil is characterized by medium acid to very acid reaction, from insufficient to elevated organic matter content, from low to very high phosphorus content, and medium calcium content. Priekuli soil is characterized by very to normal acid reaction, insufficient to optimal organic matter content, and low to medium calcium content.
In all of the soil samples found in both Stende and Priekuli, sulphur content was very low. According to research results, the optimal (sulphate) sulphur content

of soil could be from 10 to 60 mg kg^{-1}, depending on organic matter in the soil.

However, soils poor in organic matter contain sulphur of less than 5 mg kg^{-1}. One explanation might be the soil fertilization trend of recent years: natural manure appears to have been replaced with manufactured phosphorous fertilizers as the European Union and other countries try to enforce measures aimed at the reduction of sulphur emissions from industrial production processes (Smit et al., 2009). It should be noted that sulphate sulphur leaches out of soil.

Results of the soil analysis show that there is a need to further study asparagine and aspartic acid content changes in rye which occur as a result of cereal growing technologies used, taking into account both fertilization systems and the agrochemical properties of agricultural lands.

Amino acid content of rye flour

Aspartic acid is an amino acid, its structure has one amino group and two carboxyl groups, but in reaction with free NH_3 forms amide of aspartic acid, or asparagine, it is known as a neutral polar amino acid. The study identified 16 amino acids in rye flour.

Table 4

Amino acids of whole grain rye flour, g 100 g^{-1}

Amino acids	Kaupo 1	Kaupo 2	Agronom 2
Valine	0.41	0.50	0.58
Leucine	0.66	0.75	0.82
Isoleucine	0.37	0.41	0.46
Phenylalanine	0.98	1.08	1.22
Lysine	2.92	2.53	2.68
Arginine	0.49	–	0.52
Aspartic acid	0.65	0.79	0.90
Serine	0.43	0.46	0.53
Glutamic acid	2.29	2.65	3.02
Glycine	0.49	0.54	0.58
Threonine	0.31	0.35	0.39
Alanine	0.36	0.54	0.62
Proline	1.00	1.13	1.31
Tyrosine	0.19	0.22	0.26
Methionine	0.11	0.10	0.10
Total:	11.65	12.05	13.97

The amino acids content of rye flour is given in Table 4. The total content in ‘Kaupo’ variety rye whole meal flour was from 11.65 to 12.05 g 100 g^{-1} but in hybrid variety ‘Agronom’ was 13.97 g 100 g^{-1}. The aspartic acid content of rye whole meal flour was from 0.65 to 0.9 g 100 g^{-1}. Literature data shows that the aspartic acid content of whole grain rye is 503 μg kg^{-1} (Mustafa et al., 2007). This indicates that the flour used in the study has higher aspartic acid content.

Rye bread baking tests for evaluating enzyme asparaginase, rosemary extract and citric acid influence on acrylamide formation. To evaluate the impact of recipes and bread baking technology on acrylamide formation, the amino acid content was measured in dough and bread, as well as acrylamide content in the bread samples**.** Obtained results are given in Table 5.

Table 5

Acrilamide content in samples of different types of rye bread

No	Description of rye bread (rye grain variety, growing place)	Acrilamide content, μg kg^{-1}
1	Rye bread baked in form (‘Kaupo’, Priekuli)	10.9
2	Rye bread baked in form (‘Kaupo’, Priekuli) with enzyme	14.9
3	Rye bread baked in form (Agronom, Priekuli)	17.4
4	Rye bread baked in form (‘Agronom’, Priekuli) with enzyme	16.4
5	Rye bread baked in form (‘Kaupo’, Stende)	12.5
6	Rye bread baked in form (‘Kaupo’, Stende) with enzyme	15.6
7	Rye bread baked in form (‘Agronom’, Stende)	16.8
8	Rye bread baked in form (‘Agronom’, Stende) with enzyme	18.2
9	Rye bread (‘Kaupo’, Priekuli) control	98.6
10	Rye bread (‘Kaupo’, Priekuli) with Rosemarie extract 0.1%	100.7
11	Rye bread (‘Kaupo’, Priekuli) with citric acid 1%	34.3
12	Rye bread (‘Kaupo’, Priekuli) with Rosemarie extract 0.5%	106.6
13	Rye bread with fruits (at production company) control	177.1
14	Rye bread with fruits (at production company) with Rosemarie extract 0.1%	238.6

In analysing amino acid content in rye dough and bread, the most attention was heeded to aspartic acid content and its changes during bread production. The aspartic acid in rye dough samples was from 0.38 to 0.48 g 100 g^{-1}. Significant distinctions between different samples and samples with or without adding enzyme asparaginase were not observed. The aspartic content of bread samples ranged from 0.32 to 0.84 g 100 g^{-1}. However, it should be noted, the relation between different samples was not observed.

The enzyme asparaginase was added to bread samples, 500 U kg^{-1} in relation to flour weight. Scientific literature indicates that the amount of added enzyme can be from 200 to 2000 U kg^{-1}, and can reduce acrylamide content up to 70% (Capuano, 2009). The bread samples baked in forms (Fig. 1) do not have dark and thick crusts, and the acrylamide content is from 10.9 to 18.2 μg·kg^{-1}. Such acrylamide content is insignificant, according to the Joint Food and Drug Administration (FDA) and World Health Organization (WHO) Expert Committee on Food and Additives (JECFA). Their recommendation is that the acrylamide content in bread should not exceed 150 µg·kg^{-1} (EFSA, 2009). The impact of enzyme action on changes in acrylamide content was not observed. Admittedly, the enzyme asparaginase is expensive, and its wider utilization in bread manufacturing is not cost effective. The evaluation of the bread quality and physical chemical indicators between different varieties of rye grains and different cultivation regions did not show significant differences. The moisture of all dough samples was similar, ranging from 48.5 to 50%. The moisture of the bread samples was from 40.2 to 41.6%.

The study evaluated the impact of a natural antioxidant and citric acid on acrylamide content and rye bread quality.

The bread samples were baked on clay, resulting in a darker and thicker crust than in samples baked in forms.

Analysis of acrylamide content in bread samples baked on clay (Table 5) show that it has increased – from 100.7 to 106.6 µg kg^{-1} in samples with added rosemary extract, and 98.6 µg kg^{-1} in the control sample. From this it can be concluded that rosemary extract added to rye bread dough, in amounts of 0.1 and 0.5% does not have significant impact on acrylamide content.

The rye bread with fruits, baked in an industrial bakery, has an acrylamide content of 177.1 µg kg^{-1}, which exceeds the recommended quantity, but the addition of 0.1% rosemary extract raises acrylamide content up to 238.6 µg kg^{-1}.

The research carried out by Ozolina (2011) concluded that bread type, baking oven, and sugar content in dough do not have significant impact on acrylamide content in bread, but that the addition of dried fruits significantly impacts the content of acrylamide in the bread (p=0.025). The sugar content is not the main reason for the presence of acrylamide in bread, as also confirmed by a similar study (Springer et al., 2003). The scientific literature has no information about the possible influence of dried fruit additives on the acrylamide content in bread and other products.

The bread samples with 1% citric acid showed an acrylamide decrease of 66%, and in the bread it is 34.3 µg kg^{-1}.

It should be noted that the sensory qualities of bread with such a large citric acid presence were lower than that of control samples because the bread taste was markedly sour. These results suggest that the presence of citric acid can reduce acrylamide content in bread, only the quantity to be added must be correctly chosen, in order to not decrease the product sensory characteristics.

Based on the acrylamide content in foodstuff, it is possible to calculate contamination intake level in the human body. If daily ingestion of whole grain rye bread is 200 g, then acrylamide intake is 9 to 10 µg. Thus the calculation for a person weighing 70 kg, is approximately 0.13 µg acrylamide per kg body weight.

Figure 1. Samples of rye bread baked in forms

According to World Health Organization information, the level of acrylamide intake from the Latvian bread used in this case is medial. Although this indicator is not high, the issue of acrylamide content and the means to reduce it are actually urgent.

Conclusions

The results of soil composition analysis indicated insufficient content of sulphur containing compounds, which significantly impacted aspartic acid and asparagine content in grains as well as on bread quality.

The results of soil analysis give evidence for further investigations necessary on asparagines and aspartic acid changes in rye in connection with technology used in cereals cultivation in Latvia, with evaluation on fertilizer systems as well as agricultural land agrochemical properties.

Baking time significantly influenced the formation of acrylamide, therefore it is critical that the optimum baking time for specific bread types is not exceeded.

The results confirm the studies of other researchers who have observed that acrylamide content in bread increases with prolonged baking time and increased temperature.

The use of the enzyme asparaginase in the production of rye bread does not significantly reduce acrylamide content.

The addition of rosemary extract to rye dough up to 0.5% does not reduce the content of acrylamide in rye bread.

The addition of citric acid (1%) to rye dough reduced the acrylamide content in bread by 66%. It is necessary to evaluate the admissible amount of citric acid in order to not change the bread sensory qualities of acidity and aroma.

Acrylamide content in rye bread significantly increases with the introduction of dried fruit into the bread.

The reduction of acrylamide content in bread should be treated as a complex system of interlinked factors: soil, grains, technological processing and bread.

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