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# Production of highly nutritious functional food with the supplementation of wheat flour with lysine

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**Abstract.** During our research, we added 0.5–2.0% L-lysine to wheat flour in order to increase the quantity of this essential amino acid and the biological value of the wheat protein, producing such a functional, health-protecting, and health-preserving foodstuff that is suitable for satisfying

**Keywords and phrases:** L-lysine, lysine supplementation, essential amino acid, biological value of protein, Maillard reaction, amino acid analysis, high-performance liquid chromatography

the lysine requirement of humans, assuming normal nutrition. Furthermore, by the increase of the biological value completing the wheat flour with a higher amount of lysine, we could produce such a functional, health-protecting and health-preserving food that is suitable for containing or preventing lysine malnutrition symptoms. During our work, we determined the quantity of the Maillard reaction products (hydroxymethyl-furfural) and the lysine content developed during the baking of the wheat flour used for bread baking and in the bread baked with supplemented or without supplemented lysine, and evaluated the sensory characteristics of the produced functional food and the bread supplemented with lysine.

# 1 Introduction

Nowadays, there is a growing interest in functional foods with therapeutic effects and an increasing number of articles on the issues of diet and health. Numerous books, periodicals, and online information have been published on the effects of functional foods and several television programmes are focused on the topic of disease prevention and treatment (*Wildman*, 2007). The functional food industry – consisting of food, beverage, and supplement sectors – is one of the several areas of food industry that has been experiencing fast growth in the recent years. The propelling force of this significant increase does not only consist of the group of health-conscious customers but it also involves certain endemic diseases inseparable from our civilized lifestyle (diabetes, diseases of the nervous and digestive system). These days, many of us are still sceptical about the beneficial effects of functional foods since in order for them to take effect we are required to consume these products on a regular basis, while their beneficial effect might take months or even years to yield the desired outcomes.

In several countries of the developed world, protein deficiency is a prevailing factor due to the predominance of low-protein plant-based diet. Protein deficiency may lead to growth retardation/delayed growth, oedema formation, and anaemia, whereas in case it is combined with energy deficiency the resulting malnutrition may cause death in many infants and young children. As a result of researches carried out in the past decades, today's subject-matter is not the protein needs in general but the specific quantitative requirements in terms of the essential amino acids. It has also been revealed that we must not only cover the deficiencies in limiting amino acids but we must also aim at achieving a balanced ratio of essential amino acids. Moreover, we must also

pay close attention to obtaining an optimal ratio of essential/non-essential amino acids in our foods.

Once the industry-like production of amino acids has started, green light is given for amino acid supplementation, during which growth will approximate the optimal value. Recently, besides data on amino acid requirements, the optimal and minimal protein levels are also provided. In case of a sufficient protein level, the energy surplus will facilitate the energy-intensive process of protein synthesis, increase weight gain, and enhance benefits and protein utilization (*Csapó* & *Csapóné*, 2007; *Csapó et al.*, 2007).

# 2 Literature review

## 2.1 Enhancing the nutritional value of wheat flour and wheat-flour-based products

The protein needs of an adult person is 80–110 g/day depending on age and the exerted physical effort. In the case of a mixed diet, this amount of protein contains sufficient essential amino acids, but a one-sided diet may result in essential amino acid deficiency even if combined with an adequate protein consumption. Essential amino acids for an adult person include the following: isoleucine, leucine, lysine, methionine, phenylalanine, histidine, tryptophan, valine, and threonine (*Csapó* & *Csapóné*, 2004). Proteins that contain all essential amino acids in a sufficient amount and in an adequate proportion are called complete proteins – such are the proteins of meat, egg, and milk. Plant-based proteins, however, lack lysine, methionine, threonine, and tryptophan to varying degrees. Since there may occur certain abnormalities in the vital functions of those consuming small amounts of complete proteins, they supplement the largely plant-based foodstuffs with the missing essential amino acids. This supplementation would be most needed in the case of cereal-based foodstuffs because the proteins of wheat and rye contain only small amounts of lysine, methionine, and threonine.

In Europe, people usually tend to opt for a supplementation with natural protein resources, for which the various soy-based products are the most suitable as soy protein contains a large amount of lysine and is stocked up with threonine above the average level. Its disadvantage is, however, the relatively low methionine content. Primarily, they used to fortify wheat-flour-based products – the efficiency of this protein supplementation is examined with the help of biological or chemical verification methods. Chemical methods can provide faster results, allowing us to determine the protein's amino acid

composition and compare it to complete proteins by the calculation of such chemical indices that give numerical information on the nutritional value of food proteins (*Csapó* & *Csapóné*, 2006).

## 2.2 The use of lysine in flour enrichment

The amount and ratio of amino acids in wheat is far from the optimal values necessary for the human organism, giving the nutritional value of wheat protein a score of approx. 53 on a scale of 100. Since there is a low proportion of lysine first of all, the high-lysine-content materials are the most suitable for the amino acid enrichment of wheat flour (potato (biological value: 73) and soy (biological value: 74–78)).

As the regular consumption of foodstuffs made from cereals is a widely used practice in our days, people have long been engaged in using trace elements (Se, Ca, Cu, Zn, Fe, P), vitamins (vitamin B family, vitamin E), proteins, and amino acids (tryptophan, lysine, threonine) in flour enrichment and fortification procedures.

Research on the clinical and dietetic application of L-lysine looks back to a history of 30–40 years. In 1976, *Titcomb* & *Juers* patented a type of bread with an amino acid composition corresponding to that of a complete protein. In producing it, besides applying various protein supplementations, L-lysine hydrochloride was added to the flour until reaching a 0.1–0.5% proportion. *Mauron et al.* (1976) added to wheat flour the ε-amino acyl derivative of lysine, which will later create lysine during deacylation in the organism. In order to achieve a balanced amino acid composition, aside from acyl-lysine, an approx. 0.1% of L-lysine hydrochloride was also added to the flour. *El-Megged* & *Sands* (1990) elaborated a procedure during which the lyophilized sample of a lysine-producing lactic acid bacteria strain was added to the leaven, thus enriching the amino acid composition.

On the average, there was a 0.2–0.5% addition of lysine to the flour in order to increase the protein content. *Figueron et al.* (2005) added 0.5% of lysine to the flour, while *Muhammad et al.* (2012) reported on a 0.2–0.3% enrichment. *Tajammal et al.* (2004) employed a 0.5% enrichment, yielding beneficial effects in children's development, increased haemoglobin level in women, and elevated levels of transferrin in men. According to *Karcz* (2004), the consumption of 325 g of bread/day is recommended to satisfy 25% of people's daily need of lysine.

*Wenhua et al.* (2004) conducted an experiment where the subjects consumed flour-based bread fortified with 0.3% of lysine, for a period of 3 months.

They found that the consumption of lysine-enriched bread has positive effects on the immune system; although the haemoglobin level does not increase, the 0.3% lysine improves the immune system due to its effect on IgA, IgB, and IgE.

Several research results confirm the beneficial effects of lysine-enriched flours: *Anton et al.* (2008) and *Mora Aviles et al.* (2007) studied the effects of bean meal supplementation in tortilla, *Tyagi et al.* (2007) investigated the effects of mustard flour in rusk, while *Lindenmeier & Hofmann* (2004) looked into how a lysine derivative affects baking properties.

## 2.3 The physiological effects of lysine

It has been known for long that in the absence of L-lysine – the essential amino acid making up our proteins –, dietary calcium cannot get to the bones and the synthesis of a number of proteins will be inhibited. L-lysine plays a fundamental role in the formation of collagen that makes up the organic substance of the bone and skin, but its effect in strengthening the immune system and combatting viruses is also confirmed.

With respect to bone metabolism and the prevention of osteoporosis, the consumption of calcium, phosphorus, and vitamin D have been for long in the focus of literature as primary nutritional factors although vitamin C and lysine are also crucial in the formation of collagen making up the organic substance of the bones. Vitamin C takes part in the activation of vitamin D, on the one hand, and converts lysine, which takes part in building up the collagen substance of the bone, into a form suitable for the creation of procollagen, on the other hand. Research findings corroborate that, besides vitamin C, L-lysine is also a major contributor to the formation of a healthy bone structure (*Civitelli et al.*, 1992).

*Pauling* (1991) reported on the beneficial effects of lysine and vitamin C on the vascular system and on coronary artery diseases. *Rath* (2001) developed a product suitable for treating diseases related to the impairment of the extracellular matrix (atherosclerosis, cancer, infection, or other inflammatory diseases). The product contained lysine, proline, ascorbate, and their derivatives and synthetic analogues as well as vitamins, provitamins, and trace elements. In their study on L-lysine and the reaction of various carbohydrates, *Kitts* and *Hu* (2005) demonstrated that Maillard reaction products show a significant antioxidant activity.

In summary, we can state that L-lysine intake in any form increases the biological value of lysine-deficient proteins, contributes to the optimal devel-

opment of the young organism, and, besides its many therapeutic effects, can be effectively applied in combatting the herpes virus.

### 2.4 The role of lysine in the formation of Maillard reaction products

Monosaccharides, reducing carbohydrates in general, with free amino group, react with each other under appropriate conditions, and by this reaction aroma compounds and brown-coloured pigments, so-termed melanoidins, are created. This process is called the Maillard reaction. In the production of lysine-content bakery goods – as the ε-amino group of lysine is extremely sensitive to the Maillard reaction –, during the non-enzymatic browning reaction conducted at an appropriate temperature, antioxidants as well as colour and flavour substances are created, which contribute to the health-protective effect (*Csapó et al.*, 2006).

In planning the composition of the ingredients, we must take account of the Lys/Arg ratio and that using any amount of lysine under 1% should be avoided in order to achieve the desired therapeutic effect, while an amount of lysine exceeding 5% may significantly spoil the product's palatability traits, as according to our pre-experiments, since the large amount of Maillard reaction products may come with unwanted flavour and colour effects (*Csapó & Csapóné*, 2006, 2007).

## 3 Aims of the experiments

The aim of our research is to produce a lysine-fortified bread as a functional food which helps in treating the symptomatic herpes simplex virus and has beneficial effects in the treatment of osteoporosis and other diseases of the circulatory system. We expect that adding a right dosage of lysine – according to our previous researches and preliminary estimates: 0.5–2% – to wheat flour will increase the amount of essential (and, in the case of wheat flour, limiting) amino acids and that adding an amount higher than necessary for increasing the biological value will result in such a functional, health-protective and health-preserving product that will help eliminate the damages caused by the herpes virus in humans.

We also hope that the compounds produced during the baking of bread, with the remainder of L-lysine, will efficiently inhibit the viral replication of herpes simplex and will have several positive characteristics due to the outstandingly high lysine/arginine ratio, which makes the bread with an enhanced

lysine content a valuable functional food. As during the baking process bread-crumb temperature does not exceed 100 °C, we were hoping for a practically unchanged lysine content inside the bread, while in the bread-crust and directly underneath of it lysine would be transformed in significant quantities into colour and flavour substances (Maillard reaction) as well as antioxidants.

In summary, the point of our solution is that we make significant changes to the proportion of lysine in relation to the complete amino acid composition, achieving therapeutic effects that will help in curing and preventing herpes besides developing further useful characteristics. Owing to the significant changes in the lysine/arginine ratio, we planned on adding 0.5–2% of lysine to wheat flour, thus obtaining a functional food of high biological value with health-protecting and health-preserving effects as well. In this article, we report on our research results regarding the lysine-fortified bread, while our future publications will give an account of the production of high-lysine-content biscuit, on investigation into lysine absorption and its anti-herpes effects.

# 4 Materials and methods

## 4.1 Combination of the basic ingredients for bread and the baking process

In planning the composition of the ingredients, we must consider the end-product's lysine content and its Lys/Arg ratio. Using any amount of lysine under 0.5% should be avoided in order to achieve the desired therapeutic effect, while an amount of lysine exceeding 2% may significantly spoil the product's palatability traits, as according to our pre-experiments, since the large amount of Maillard reaction products may come with unwanted flavour and colour effects.

The composition of the raw mixture corresponds with the composition of the similar but lysine-free product. L-lysine was added to the flour in the form of L-lysine-hydrochloride, in a solid, powdery state, and then mixed with the other ingredients and water, in accordance with bread baking technology requirements. To determine the optimal amount of lysine, it was added to the wheat flour in 0.5, 1, 1.5, and 2% concentrations, followed by the examination of all properties of the obtained breads, in compliance with the standard practical procedures used in the qualification of bread.

During the baking of white bread, we complied with the general principles, and made use of the following ingredients: 205 ml water, 20 ml oil, 15 g salt, 20 g skimmed-milk powder, 350 g flour, and 10 g dry yeast. Depending on the

nature of the desired product, the dough was leavened, shaped, flavoured, and then baked at 210±10 °C in the automatic bread baking machine operated at our Department. With the preliminary results in hand, we can adjust baking temperature and time as required, depending on whether we intend to produce a bread with higher lysine or higher antioxidant content.

## 4.2 Analytical investigations

### 4.2.1 Determination of total protein and lysine content

The total protein content of the obtained breads was determined with the help of Velp Scientifica UDK 159, an apparatus operating on the principle of the Kjedahl method. To measure the amount of lysine, we applied the high-performance liquid chromatography method with orto-phthalaldehyde (OPA)-2-mercaptoethanol pre-column derivatization. Apparatus used: Varian Pro Star; method applied: fluorescence detection (λex=340 nm, λem=455 nm); column: Pursuit C18 5μm, 250×4.6 mm; gradient elution: A: 100 mmol/l acetate puffer (pH=6.95) 925 ml, methanol 50 ml, tetrahydrofuran 25 ml, B: methanol 975 ml, tetrahydrofuran 25 ml; flow rate: 0.8 ml/min; gradient program for the analysis: 0–8 min; A component 100%, B component 0%, 8–11: 90–10, 11–16: 75–25, 16–20: 60–40, 20–24: 60–40, 24–27: 40–60, 27–32: 0–100, 32–33: 0–100, 33–47: 100–0. Under the conditions employed, there was a fairly clear separation of lysine from the other amino acids, while quantification was not disturbed by any circumstances.

### 4.2.2 Determination of HMF

In order to estimate the amount of Maillard reaction products, high-performance liquid chromatography was used to determine the hydroxy-methyl-furfural (HMF) concentration of the bread. For the measurement of HMF, samples taken from the different types of bread were ground in electric mill to 0.75 mm sieve size. An amount of 1 g was taken from each sample, to which we added 9 ml of de-ionized water, and then we had it mixed with a Vortex tube mixer. Following this, with a view to eliminate any interfering substances, 0.5 ml of Carrez I and Carrez II solution is added to the extract, and then centrifuged for 10 minutes at 5,000 rpm. The supernatant was removed and filtered through 0.45 μm filtration hoods, and 20 μl of the obtained solution was injected into the Varian Pro Star HPLC apparatus. Isocratic method was used to measure HMF, where the mobile phase was a 20:80% mixture of 5% methanol-acetic acid, the stationary phase was Pursuit C18 column (250×45 mm), the rate

flow was 1ml/min, and UV detection was performed at a wavelength of 285 nm.

### 4.3 Sensory analysis

Bread samples were subjected to sensory analyses that made use of a questionnaire compiled according to the following criteria:

1. Shape: typical of bread (loaf shape), regular, proportionately convex, not deformed.

2. Crust: typical of the particular type of bread; shiny, smooth or cracked; scattered and/or sliced; not split all the way, not sooty/soiled/burned/ saturated/damaged.

3. Internal content: baked thoroughly; does not separate from the crust; the colour is typical of the flour used; the substance is consistent, flexible, and free of lumps; not lardy, sticky, crumbly, or falling apart; does not contain foreign substances and is not damaged by microorganisms.

4. Taste and aroma: aromatic – typical of the particular type of bread, not having strange taste or odour (*Codex Alimentarius*, 2004).

After cooling, the control bread and the lysine-fortified breads were examined for shape and crust by 20 survey participants. In what followed, we prepared slices of 20–25 g from each sample, containing both crumb and crust parts. Based on these, they compared the breads in terms of internal content, taste, and aroma. Participants could provide a score between 1 and 5 for each of the five properties in the evaluation of which we also made use of weighting factors. The sum of the factors was 4 in each case; thus, the highest possible score for the five property groups altogether was 20. The weighting factors for the individual properties were as follows: shape – 0.6, crust – 0.6, internal content – 1.4, aroma – 0.4, and taste – 1.0.

Sensory analyses began with the inspection of the uncut product. We established the morphological properties and that whether the product volume and mass appear to be proportionate. Crust properties included surface characteristics, colour, crust shine, structure, and substance. Crust thickness must be examined in the sliced product and the consistency of the internal content should also be inspected. Examination of the properties concerning the internal content must be performed on the freshly cut product: it must be established whether the colour is appropriate for the particular product and

if it is sufficiently consistent throughout the product. The structure of the internal content should be examined by palpation as well on a slice cut out from the central part of the bread. The slice of bread must be at first gently and then more strongly pressed to check for softness and rigidity. Flexibility is examined in the same manner. To establish glutinosity and crumbliness, one must run their fingers over the surface of the slice, and then observe the amount of crumbs created. Glutinosity can be determined by the degree of cohesion.

Aromatic properties were observed as follows: pressure was applied several times on the cut-surface of the halved end-product, and then we had a smell of the volatile substances from the inside. When analysing taste, we must first establish whether it is appropriate for the particular product. We must also find out if any of the following can be observed besides the taste specific of the product: strange, excessively salty/unsalted or sweet taste; too sour/bitter; detection of foreign materials, uncharacteristic of the product, while chewing. At the same time with tasting, we can also feel its substance; therefore, this method helps us determine not only the properties of the internal content but also those of the crust.

### 4.4 Statistical evaluation of the data

We used Microsoft Excel 2010 software package and one-way analysis of variance to perform the statistical evaluation of the data.

## 5 Results and discussion

### 5.1 Total protein content of the bread

To determine the total protein content, the samples taken from the different types of bread were ground in electric mill to 0.75 mm sieve size, and then an amount of 2 g was taken from each sample and digested. Digestion and distillation of ammonia were equally repeated three times. We measured the following amounts of protein content: control bread – 8.86±0.18%, bread made from 0.5% lysine-enriched flour – 9.20±0.065%, bread made from 1% lysine-enriched flour – 9.24±0.038%, bread made from 1.5% lysine-enriched flour - 9.67±0.037, and bread made from 2% lysine-enriched flour – 10.14±0.023%.

We used one-way variance analysis to demonstrate that the added lysine significantly increases the total protein content of the control bread, but this increase is not such as we would expect from the amount of the added ly-

sine, what can be explained with the partial decomposition of lysine and the development of the increased amount of Maillard reaction products.

### 5.2 Lysine content of the bread

In order to determine lysine content, bread proteins were hydrolysed with 6 M HCl at 110 °C for a period of 24 hours; then, after appropriate dilution and derivatization, 20 µl of the hydrolysate was injected into the high-performance liquid chromatography. We measured the following amounts of lysine content: flour – 0.32±0.05, control bread – 0.38±0.06, bread made from 0.5% lysine-enriched wheat flour – 0.48±0.12, bread made from 1% lysine-enriched wheat flour – 0.67±0.14, bread made from 1.5% lysine-enriched wheat flour – 0.95±0.13, and bread made from 2% lysine-enriched wheat flour – 1.11±0.13%. One-way variance analysis showed significant differences.

As a result of the additives' effect, the lysine content of the flour increased from 0.32% to 0.38%. The added lysine yielded necessarily an increased lysine content in the bread although a certain amount of loss could also be observed. Lysine loss can be explained by the lysine transformation (Maillard reaction) that took place in the bread-crumb, but mostly in the crust during baking. This loss is most striking in the case of the bread made from 2% lysine-enriched wheat flour, where we measured an amount much less than expected.

### 5.3 Amount of the hydroxy-methyl-furfural

We measured the following amounts of HMF content: control bread – 2.01±0.05 mg/kg, bread made from 0.5% lysine-enriched wheat flour – 3.25±0.12 mg/kg, bread made from 1% lysine-enriched wheat flour – 4.82±0.07 mg/kg, bread made from 1.5% lysine-enriched wheat flour – 5.30±0.15 mg/kg, and bread made from 2% lysine-enriched wheat flour – 48.02±0.22 mg/kg. Although the one-way variance analysis of the data showed significant differences, meaning that the added lysine resulted in an increased content of hydroxy-methyl-furfural in the bread, a considerable amount was measured only in the case of the 2% lysine-enriched wheat flour.

### 5.4 Sensory analysis results

Analysis results are included in *Table 1.*

Regarding shape, we hardly found any difference between control bread and breads made from 0.5–1.5% lysine-enriched wheat flour, as the respective scores ranged between 4.0 and 5.0. It came as a surprise that the score for

the bread made from 1.5% lysine-enriched wheat flour dropped to 3.2 after an additional 0.5% lysine-enrichment. Similar tendency could be observed in the crust although with much less differences. We found no such correlation in the case of the internal content and, what is more, it appears that in the case of the 2% lysine-enriched wheat flour lysine supplementation improved internal content as compared to control. Lysine supplementation did not have a significantly adverse impact on the aromatic properties either, but, in fact, breads made from 0.5–1.5% lysine-enriched wheat flour were assigned a higher score as compared to control and breads made from 2.0% lysine-enriched wheat flour.

Table 1: Arithmetic mean of the scores assigned to breads

| *Bread type* Rated properties | Control bread | Bread made from 0.5% lysine-enriched wheat flour | Bread made from 1% lysine-enriched wheat flour | Bread made from 1.5% lysine-enriched wheat flour | Bread made from 2% lysine-enriched wheat flour |
|---|---|---|---|---|---|
| Shape | 4.2 | 4.6 | 4.0 | 5.0 | 3.2 |
| Crust | 4.2 | 4.4 | 3.8 | 4.6 | 3.7 |
| Crumb | 3.6 | 3.2 | 4.2 | 4.8 | 4.5 |
| Aroma | 3.0 | 4.7 | 4.2 | 4.9 | 3.1 |
| Taste | 4.7 | 4.7 | 4.9 | 5.0 | 1.0 |

With respect to the examined properties, we obtained the most unexpected results in the case of taste. It appears that the taste of bread remains practically unchanged until reaching a lysine supplementation of 1.5%; moreover, this latter case yielded the highest score. Nevertheless, in the case of the 2% lysine fortification, the average score of 4.8 plummeted to 1.0 and the bread gained a bitter taste, therefore becoming unpalatable.

We multiplied the scores for bread in *Table 1* with the weighting factors, and obtained the following values:

Table 2: Scores for the different types of bread Bread types

| Bread type | Control bread | Bread made from 0.5% lysine-enriched wheat flour | Bread made from 1% lysine-enriched wheat flour | Bread made from 1.5% lysine-enriched wheat flour | Bread made from 2% lysine-enriched wheat flour |
|---|---|---|---|---|---|
| Achieved score | 15.98 | 16.46 | 17.14 | 19.44 | 12.68 |

Multiplying the scores with the weighting factors led to similar conclusions. As an effect of 5% lysine supplementation, the 15.98 score of control bread increased to 16.46, after 1% lysine supplementation, it went up to 17.14, after 1.5% lysine supplementation to 19.44, while after 2% lysine supplementation, due to the extremely low score given for taste, it dropped to 12.68. Data in *Table 2* suggest that the highest score was achieved by the bread made from 1.5% lysine-enriched flour, which leads us to the conclusion that this type of bread disposes of the most favourable sensory properties. Bread made from 2% lysine-enriched flour received the lowest score due to its strong bitter taste; its crust had a much darker colour in comparison with other breads and its aroma score was also very low: 3.1. According to sensory analysis results, 1.5% lysine supplementation enhances the palatability traits of the bread, namely its taste and colour, but it also affects the development of its internal content in a positive way.

## 6 Conclusions

Performing the sensory analysis, we concluded that the enrichment of wheat flour with 0.5–1.5% lysine either improved sensory properties or did not change them at all as compared to control bread, but breads cannot be enriched with a proportion exceeding 1.5% since that would increase the concentration of the Maillard reaction products, which has a negative influence on the taste factor. Besides taste, bread-crust gains a much darker shade, which is undesirable for customers. The hydroxy-methyl-furfural content of the bread made from 2% lysine-enriched flour is much higher than that of the breads with a lower amount of lysine supplementation – therefore, we put down the appearance of the strong bitter taste to this fact.

Considering our future research work, the next step is to examine the amount utilized from the added lysine, have a look into the changes in the lysine/arginine ratio of the obtained breads, and study whether lysine produces therapeutic effects in such concentration. Descriptions found in literature did not include studies on breads made from flour with an added lysine content higher than 0.6%, wherefore our results could not be compared with literature data. Lysine supplementation of 1.5% or less enhances the palatability traits of bread and yields a functional food with a balanced amino acid composition and higher biological value, while it might also produce therapeutic effects.

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# Production of high-lysine-content biscuit and examination of the absorption of lysine in humans

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**Abstract.** In the Medical and Health Centre of the University of Debrecen, we examined the changes in the free amino acid content of the blood serum of control and experimental individuals after consumption of 2,000 mg of lysine-laden biscuits. We baked the biscuits at 130 °C, during which the greater part (70–75%) of the lysine was not converted into Maillard reaction products. After 30–60 minutes of consumption of the biscuits, the free lysine content of the blood serum increased significantly in the experimental and control group with 41–46%, and even

**Keywords and phrases:** L-lysine, biscuit supplemented with lysine, Maillard reaction, amino acid analysis, absorption of lysine, ratio of lysine/arginine, herpes simplex virus (HSV), effect against herpes

after three hours of consumption the level was 20% higher than in the initial concentration. The free arginine content of the blood serum did not change after the consumption of control and lysine biscuits neither in the control nor in the experimental group. Therefore, the free lysine/free arginine ratio of the individuals consuming lysine increased significantly compared to the initial and the control group's value. The antioxidant level of the blood serum in the control group remained unchanged after the consumption of the control biscuit, while in the case of the experimental individuals who consumed lysine-fortified biscuits it increased by 40–45% compared to the initial level. Summing up: After consumption of the biscuits with 2,000 mg of free lysine, the concentration of free lysine in the blood serum, its free lysine/free arginine ratio and antioxidant level increased significantly. Our researches have clearly demonstrated that the active substances of the biscuit got into the blood serum, so the investigation of the active substance and the evaluation of the physiological effects are definitely recommended in the long run.

# 1 Introduction

Lysine (also known as 2,6-diaminohexanoic acid, or $\alpha, \varepsilon$-diamino caproic acid) is one of the twenty protein-building amino acids, perhaps the most important essential amino acid, which also plays the role of limiting amino acid in most plant foods (except for leguminous plants), since compared to human needs it can be found in the smallest proportion. It is essential for humans and all farmed animals; the daily human need is 1.0–1.5 g, which can be satisfied only with the combined consumption of appropriate animal and plant proteins. Cereals regularly consumed by humans (wheat, rice, or maize) as well as foodstuffs made from them are deficient in lysine; among frequently consumed plant-based proteins, soy protein has a relatively high lysine content (*Csapó & Csapóné*, 2004). The estimated lysine needs – as according to WHO recommendations – of infants, young and school children, and adults are outlined in *Table 1.*

Table 1: Estimated lysine needs (mg/BWT/day) based on WHO recommendations

| Amino acid | Infants (3–4 months) | Young children (2 years) | School children (10–12 years) | Adults |
|---|---|---|---|---|
| Lysine | 103 | 64 | 44–60 | 12.0 |

BWT = body weight (kg)

In many countries of the developing world, lysine deficiency is a prevailing factor due to the predominance of low-protein plant-based diet. Lysine supplementation of wheat proteins was first practised in the mid-50s, though with little success due to the legal regulations of that period (*Flodin*, 1997). Lysine supplementation of animal feeds has been a daily practice in Hungary since the late 70s, made necessary by the general use of intensive livestock farming. The annual worldwide use of synthetic lysine in animal nutrition is reported to be of 800,000 tons, nowadays regulations also providing for food supplementation (*Toride*, 2007).

Besides its many biological functions, we must highlight that lysine takes an active share in treating the symptoms of the herpes simplex virus (HSV). To make use of this functionality, our intention was to introduce a significant amount of lysine into the human organism to monitor through our experiment how it becomes effective and gets absorbed, whether it has therapeutic benefits, and if so, then to have a look at its efficiency in preventing the development of herpes as well as in the treatment of the already developed disease.

# 2 Literature review

## 2.1 Absorption and therapeutic effects of lysine in combating HSV

Experiments in human nutrition have demonstrated that absorption from the digestive system of synthetic lysine and that of lysine with protein intake is practically the same. In 5–7 hours following consumption, lysine rapidly finds its way to the muscle tissues, where both its intra- and extracellular concentration becomes higher than in any other tissue. Lysine is an antagonist of arginine which is absolutely necessary for HSV reproduction (*Griffith et al.*, 1981). Lysine inhibits arginine absorption from the small intestines, its reabsorption in the kidney, and its transport through the plasma membranes, whereas in *in vivo* experiments it has been shown to inhibit arginine's HSV-growth-stimulating effect. The increase of the lysine/arginine ratio is crucial in combating HSV.

L-lysine has been proven in several experiments as effective in treating symptomatic herpes simplex virus, while both in *in vivo* and *in vitro* experiments it has exhibited herpes-fighting properties. *Kagan* published his results as early as in 1974, pointing out that the administration of L-lysine suppresses the viral replication of herpes simplex, allowing of an effective treatment of herpes. *Thein & Hurt* (1984) carried out an experiment with 26 participants

predisposed to develop HSV. The experimental subjects were consuming 1,000 mg of L-lysine throughout a year, causing the free lysine level of the serum to increase to 165 nmol/ml – whenever this level was maintained or increased due to the continuous administration of lysine, the incidence rate of herpes would significantly decrease in comparison to control.

Based on a survey questionnaire, *Ferroli et al.* (1996) established that more than half of the patients suffering from herpes would have a significantly improved quality of life following the regular consumption of lysine-containing products. *Richardson & Pearson* (2003) patented products in the form of tablets, creams, and solutions, containing L-lysine, zinc, selenium, copper, and cysteine, all of them found suitable for treating symptomatic HSV.

Due to space limitations, we have touched upon the major factors alone regarding the role of lysine in treating herpes, but the following studies also confirm its anti-herpes effects: *Algert et al.* (1987), *Armstrong & Elenbaas*, (1983), *Ayala & Krikorian*, (1989), *Benmohamed et al.* (2005), *Boutell et al.* (2003), *Brandimarti & Roizman*, (1997), *Digiovanna & Blank*, (1984), *Digiovanna et al.* (1985), *Ferroli et al.* (1996), *Griffith et al.* (1978, 1981, 1987), *Ishihara et al.* (1989), *Kagan* (1974, 1983), *Luo & Aurelian*, (1992), *Masterson* (1986), *McCune et al.* (1984), *Milman et al.* (1980, 1987), *Park et al.* (1982), *Ruyechan & Olson*, (1992), *Simon et al.* (1985), *Smirnova et al.* (1999), *Thein & Hurt*, (1984), *Tomblin & Lucas* (2001), and *Walsh et al.* (1983).

## 2.2 Is overconsumption of lysine possible?

The question may arise as to whether the overconsumption of lysine could cause any problems in the human organism. Researches have demonstrated that there is no threat of overconsumption either under normal nutritional conditions or in cases of lysine supplementation. Researches suggest that the prevention of HSV requires a daily consumption of 500–3,000 mg of lysine, but for maintaining a steady level 500–1,000 mg seem to be sufficient, while 3,000 mg should be applied in severe cases exclusively and for short periods of time (*Meredith et al.*, 1986; *Duncan et al.*, 1996). If, however, an amount above the necessary level would enter the human organism, it would be used up as energy. The degradation of lysine is a highly complex process since four of its six carbon atoms take part in acetoacetyl-CoA formation (ketogenic amino acid), while the other two are converted into carbon dioxide as a consequence of decarboxylation. Unlike in the case of other amino acids, removal of the ε-amino group is particularly problematic here as there is no suitable enzyme to

perform this task. There are two known routes for the removal of the ε-amino group: one of them involves the formation of cyclic intermediates (piperidine carboxylic acids, pipecolate) and the other one the formation of saccharopine in the liver by condensation of the ε-amino group and α-ketoglutarate. At the intersection of these two routes, lysine is converted at first into aminoadipate-semialdehyde and then into acetoacetyl-CoA (*Csapó & Csapóné*, 2007).

In summary, we can say that L-lysine intake, in any form, improves the biological value of lysine-deficient proteins, contributes to the optimal development of the young organism, and – besides its many other therapeutic effects – can be efficiently used in combating the herpes virus.

# 3 Research objectives

The abovementioned facts and explanations are very convincing in that lysine supplementation can be extremely useful in the prevention of many diseases as well as in their treatment in case they have already developed. The question arises, then, as to in what form lysine should be introduced into the organism. Intake in the form of medicines or medicinal preparations is problematic and is subject to special licenses, whereas the marketing of functional foods with medicinal properties has no limitations. Setting out from the above, we have decided to deliver lysine into the organism in the form of biscuit and examine how lysine is absorbed in the human body and how it increases the free lysine content of the blood serum. Our secondary aim was to look into how the duration and temperature of baking influences the utilization of lysine in the body.

# 4 Materials and methods

## 4.1 Research location

We conducted our examinations in the Medical and Health Centre of the University of Debrecen, in compliance with the laws and regulations applicable in Hungary and in possession of the licences for experimentation on humans. The Institution has at its disposal all resources in staff and equipment necessary for carrying out a Phase II.a. human experiment. Examination of the free lysine content of the biscuit and blood serum was performed at the Department of Food Science, Faculty of Miercurea Ciuc, Sapientia Hungarian University of Transylvania.

## 4.2 The product under study

For our study, we made use of the *Detki* biscuit, previously prepared with lysine supplementation, having the following major properties: lemon-flavoured calcium source, increased lysine content, and tested antioxidant activity – in the production of the *Detki Keksz Édesipari Kft.* The product contained the following ingredients: 63.4% wheat flour, vegetable fats, sugar, isosugar, whey powder, 3.8% L-lysine hydrochloride, gluten, 0.8% calcium-carbonate, aromas, raising agents (ammonium-hydrogen-carbonate, sodium-hydrogen-carbonate), antioxidants, tartaric acid, soya lecithin as emulsifier, salt, and it may contain traces of nuts, peanuts, and egg-powder. Biscuit ingredients per 100 g of product were: caloric value: 1,955 kJ, protein: 8.9 g, carbohydrate: 67.6 g – containing sugar: 21.1 g, fat: 17.5 g – containing saturated fatty acids: 8.2 g, dietary fibre: 0.2 g, sodium: 17 mg, calcium: 400 mg, L-lysine: 3,000 mg – containing free L-lysine: 2,300 mg, antioxidant activity: 76.5 mg vitamin C equivalent determined with FRAP method.

## 4.3 Baking time and temperature combinations in the production of biscuit

Since the baking process of biscuit takes up 30 minutes on average, we did not change the baking time, only the temperature in the production of the biscuit. Baking was performed at 120, 130, 140, 150, 160, 170, and 180 °C, each time followed by an examination of the biscuit composition – more specifically its free amino acid and total amino acid content, paying particular attention to free lysine – and its antioxidant activity, determined with FRAP method and expressed in vitamin C units (mg/kg).

## 4.4 The study protocol of lysine absorption

Volunteers selected for the clinical experiments had to undergo medical examinations prior to participation in the study. During these examinations, their state of health and physical condition was measured, their dietary habits were assessed via questionnaire-based interviews, and certain necessary blood tests were also carried out. Afterwards, six participants consumed six 100 g biscuits per head, each portion containing 333 mg of lysine hydrochloride; thus, the total lysine consumption amounted to 2,000 mg at the beginning of the experiment. At the same time, two further participants consumed six biscuits likewise, but without lysine supplementation – they formed the control group. Blood was drawn and the composition of these blood samples was determined

in the members of both groups immediately before consuming the biscuits and then after 15, 30, 60, 120, 180, and 240 minutes. Following centrifugation, blood plasma was divided into three parts, and its antioxidant, lysine, and calcium content was determined.

### 4.5 The employed analytical methods

To determine the antioxidant level of the blood plasma, we used the FRAP (Ferric Reducing Ability of Plasma) method, during which we added 500 µl of FRAP reagent to 100 µl of blood serum and measured light absorption at 593 nm, from which we calculated the vitamin C antioxidant equivalent.

Concentration of free amino acids in the blood plasma was performed according to *Csapó et al.* (2008). In doing so, we centrifuged the blood samples, precipitated the proteins with trichloroacetic acid, and determined the free amino acids of the protein-free solution with INGOS AAA-400 amino acid analyser, using post-column ninhydrin derivatization. Derivative absorbance was measured at 440 nm (proline and hydroxyproline) and at 570 nm (all other amino acids) and, besides lysine, all other amino acids were also determined by comparison with the standard chromatogram. In determining the calcium content of the blood plasma, we took 200 µl of it and digested it with a mixture of 1 ml concentrated $HNO_3$ and 0.5 ml concentrated $H_2O_2$ for 30 minutes at 80 °C. The final digest was diluted to 10 ml with deionized water, and the concentration of the solution was determined with Thermo Iris Intrepid II inductively coupled plasma optical spectrometer.

## 5 Results and discussion

### 5.1 Total and free amino acid content of the high-lysine-content biscuit

The total amino acid content is characteristic of wheat and of the components added to wheat flour during biscuit production, except that the added lysine has slightly changed the proportions. We measured a 2.98% lysine content in the high-lysine-content biscuit, which amounted to 24.5% in terms of protein. Apart from lysine, owing to the ratio of the components, glutamic acid (26.3%) and proline (8.0%) were also highly prevalent in the protein. Part of the 2.98% lysine is the initial lysine content of the wheat, and the other part comes from the added lysine. We measured a 0.37% arginine content in the high-lysine-content biscuit, which adds up to 3.0% in the protein, indicating that the

high-lysine-content biscuit has a lysine content almost 9 times higher than its arginine content.

The examination of the free amino acid content concluded that the 3,000 mg added lysine has dropped to 2,300 mg – 700 mg have decomposed or transformed during the baking process. Lysine is a component of the Maillard reaction, during which various coloured products (brown pigments) and aroma compounds are created by the reaction of protein and carbohydrates. Reaction stages are realized through complex Schiff-base formation, Amadori and Heyns rearrangements, dehydration and deamination steps, and Strecker degradation. The end-products of the reaction series are the coloured melanoidins and the hydroxymethyl-furfurol (HMF) capable of further reactions, while antioxidant compounds are also created in small quantities. Although these transformations reduce the available amount of lysine, they are useful for causing the formation of colour, flavour, aroma substances, and antioxidants as well.

According to our studies, 60–80% of L-lysine remained unchanged in the biscuit during the 30-minute baking process at 130 °C, resulting a lysine/arginine ratio in the end-product that is suitable for therapeutic purposes. The converted lysine created antioxidants, which enhanced the therapeutic benefit and the palatability traits of the product. We have established that the mono- and disaccharides significantly affect the amount of the resulting antioxidants, on the one hand, and that using fructose and dextrose, besides sucrose, is beneficial for this purpose, on the other hand.

The amino acid composition of the high-lysine-content biscuit as functional food shows a significant difference from that of the control biscuit. As an effect of lysine supplementation, the free amino acid content of the biscuit increased to 2.19% as compared to the control's 0.0034%, the total lysine content increased from 0.13% to 2.82%, while in the protein from 1.6% to 25.8%. The lysine/arginine ratio increased from 0.36 to 55.58 in the free amino acid fraction, whereas in terms of the total lysine content from 0.30 to 4.78. These findings are extremely important since a lysine/arginine ratio below 1 is a favourable condition for the viral replication of herpes, whereas values above 2–4 will have a therapeutic effect. The approx. 100 mg free lysine content of the 5-g biscuits can secure the 1–3 g/day lysine intake necessary to achieve the desired effect, this implying the consumption of 10–30 biscuits. The anti-herpes effect of the obtained products was verified with double-blind clinical trials.

We have established that baking temperature and time have a significant influence both on the amount of free lysine left in the product and on the

amount of the resulting antioxidants. 30 minutes of baking time at 120 °C left 95% of the lysine in free form, while this proportion dropped below 20% at 180 °C. Antioxidant activity varies inversely to this, as measured in vitamin C equivalent this value barely exceeds 0 at 120 °C, whereas at 180 °C it can reach as much as 600–700; however, in the control biscuit, this value range was 0–150. Extending baking time from 15 to 60 minutes increased antioxidant activity from 20–25 to approx. 120–140 in the 1%-lysine-content biscuit, while this value ranged between 15 and 50 in the control biscuit. In view of the available measurement data, we have optimized the technical parameters (baking time and temperature) for the conversion of high antioxidant and low lysine content.

## 5.2 Changes in the lysine content of blood serum samples

In the two control individuals, the free lysine content of the blood plasma increased from 2.80–3.00 mg/100 ml to 2.95–3.13 mg/100 ml in a period of half an hour, and then decreased to the average value of 2.55–2.88 mg/100 ml. The free lysine content of the experimental subjects' blood serum ranged between 2.77 and 3.98 mg/100 ml before the consumption of the high-lysine-content biscuit – these values increased to 3.62–5.11 after half an hour and to 3.84–6.05 after an hour, followed by a significant drop in all individuals by the end of the fourth hour. Values measured after half an hour and after an hour respectively have increased one and a half times as compared to the initial measurements, while control individuals showed virtually no such changes at all.

Table 2: Average amount of free lysine concentration in the blood serum of experimental and control subjects in function of the period after consuming the biscuits as compared to initial values

| Free lysine in blood | Time lapsed after consuming the biscuits (hour) | | | | |
|---|---|---|---|---|---|
| plasma/initial value (1.00) | 0.5 | 1 | 2 | 3 | 4 |
| Experimental subjects | 1.41 | 1.46 | 1.36 | 1.19 | 1.08 |
| Control subjects | 1.05 | 0.94 | 0.89 | 0.91 | 0.96 |

We used one-way analysis of variance to demonstrate that the consumers of Liziner had a significant increase in their free lysine level in the blood serum, while we could not detect any significant change in the case of the control group.

### 5.3 Changes in the arginine content and free lysine/free arginine ratio of blood serum samples

We have observed 2.51–3.88 mg/100 ml and 1.88–4.39 mg/100 ml of free arginine content in the control and in the experimental individuals respectively, suggesting considerable differences between the subjects. In function of the time lapsed after consuming the biscuits, the free amino acid content did not change in a statistically verifiable manner – thus, practically it remained constant throughout the experiment. We can make similar statements in terms of all other amino acids, whose concentration showed changes to varying degrees during the experiment, but we could not demonstrate significant differences between the experimental and the control group.

Then, we studied the blood serum's free lysine/free arginine ratio in the blood plasma, and established that this ratio was practically the same in the experimental (0.91–1.29) and control subjects (0.85–1.15). While the ratio remained unchanged in control subjects, experimental individuals showed increasing tendencies as follows: 1.38-fold increase after half an hour, 1.35-fold after an hour, 1.29-fold after two hours; 1.19-fold after three hours, and 1.14-fold after four hours (*Table 3*).

Table 3: Average ratio of free lysine/free arginine content in the blood serum of experimental and control subjects in function of the period after consuming the biscuits as compared to initial values

| Free lysine/free arginine ratio as compared to initial value (1.00) | Time lapsed after consuming the biscuits (hour) | | | | |
|---|---|---|---|---|---|
| | 0.5 | 1 | 2 | 3 | 4 |
| Experimental subjects | 1.37 | 1.35 | 1.29 | 1.19 | 1.14 |
| Control subjects | 0.98 | 0.97 | 0.87 | 1.03 | 0.99 |

We used variance analysis to find out that the free lysine/free arginine ratio in the blood serum of those consuming Liziner has significantly increased compared to both the initial value and the control group. The control group did not show any significant change regarding this ratio in the function of time.

## 6 Changes in the antioxidant level of blood serum samples

The antioxidant level of the control individuals' blood serum ranged between 31.2 and 37.7 mg vitamin C equivalent/1000 ml in one case and between 59.5 and 68.3 in the other case. Relevant differences could be observed between

experimental subjects as well in terms of antioxidant level (36.0–117.7), wherefore we could not detect significant differences in this respect between the two groups. However, when we did not take antioxidant level per se but studied it in its relation to the initial value, we could state that this level had significantly increased in the case of high-lysine-content biscuit consumers (*Table 4*).

Table 4: Average antioxidant level – determined with FRAP method – in the blood serum of experimental and control subjects in function of the time lapsed after consuming the biscuits

| Antioxidant activity (mg vitamin C/1000 ml) as compared to initial value (1.00) | Time lapsed after consuming the biscuits (hour) | | | | |
|---|---|---|---|---|---|
| | 0.5 | 1 | 2 | 3 | 4 |
| Experimental subjects | 1.36 | 1.41 | 1.35 | 1.45 | 1.40 |
| Control subjects | 0.96 | 1.00 | 1.11 | 1.04 | 1.07 |

## 7 Conclusions

In the Medical and Health Centre of the University of Debrecen, we examined the changes in the free amino acid content of the blood serum of control and experimental individuals after consumption of high-lysine-content biscuits. We opted for a baking temperature of the biscuit (130 °C) with a view to leaving the greater part of lysine in free form and converting only a smaller part (20–25%) through the Maillard reaction. By changing baking temperature between 120 and 180 °C, we were able to change the concentration and ratio of free lysine and of the Maillard reaction products, but since our aim was to increase the lysine level in the blood serum we kept to a temperature of 130 °C. We set the free lysine content of the biscuit to 3% so that the 5-g biscuit would contain 100 mg of lysine: this way, we could work out that a daily consumption of 10–30 biscuits would secure the necessary amount of lysine intake in order to achieve the desired therapeutic effect. 30–60 minutes after the consumption of 2,000 mg of free lysine, the free lysine content of the experimental group's blood serum showed a significant increase of 41–46% in comparison with the initial value, yielding a 20% higher concentration in relation to the initial measurement even after three hours have passed following consumption. After the consumption of control biscuits, the free lysine concentration increased in the control subjects' serum, but this was not a significant change.

The free arginine content of the blood serum did not change either in control or in experimental subjects following the consumption of the control and the

high-lysine-content biscuit respectively. As a consequence, the free lysine/free arginine ratio in the blood serum of the individuals consuming lysine increased significantly compared to the initial value and to the control group alike.

The antioxidant level of the control subjects' blood serum has remained virtually unaffected by the consumption of control biscuits, whereas in the case of experimental individuals consuming high-lysine-content biscuits this level has increased by 40–45% in relation to the initial value.

In summary, we may conclude that after the consumption of biscuits with 2,000 mg of free lysine the concentration of free lysine in the blood serum, its free lysine/free arginine ratio and antioxidant level increased significantly. Our researches have clearly demonstrated that the active substances of the biscuit got into the blood serum, so the investigation of the active substance and the evaluation of the physiological effects are definitely recommended in the long run.

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# Production of a high-nutritional-value functional food, the Update1 bread, with the supplementation of the wheat flour with high-protein-content raw food materials

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**Abstract.** During our research, we added extracted soya bean meal, egg-white powder, gluten, wheat sourdough, and bamboo fibre to wheat flour in order to increase the quantity of the essential amino acid and the biological value of the wheat protein, producing such a functional, health-protecting, health-preservative food product which is suitable to satisfy the essential amino acid requirements of humans, assuming normal nutrition. Furthermore, we could produce such a food, which, on

**Keywords and phrases:** defatted soya bean meal, egg-white powder, gluten, supplementation, essential amino acids, biological value of protein, Maillard reaction, amino acid analysis, fortified bread with high essential amino acid content

the one hand, was suitable to confine or prevent the essential amino acid's malnutrition symptoms, while, on the other hand, when applied alone, to meet the consumers' needs. During our work, we determined the protein content and amino acid composition of the wheat flour, of the additives used in bread baking, and in the bread both baked with supplementation (Update1 bread) and without supplementation (normal bread), as well as the quantity of the Maillard reaction products (hydroxymethylfurfural). We calculated the biological value of the protein of different breads and evaluated the sensory characteristics of the produced functional food and the fortified bread, supplemented with high essential-amino-acid-containing additives.

# 1 Introduction

Nowadays, there is an ever-growing interest about high-biological-value functional foods, while more and more articles are published in the groves of specialized literature on the impact of these functional foods regarding humans' diet and health. A great number of books, journals, and Internet sources treat this topic, and plenty of television programmes tackle the problem of disease prevention and treatment ( *Wildman*, 2007). In recent years, the industrial sector of functional foods – including foods, beverages, and related and supporting sectors – has become one of the fastest growing branches of food industry. The drive of this sharp increase is fuelled not only by the group of health-conscious consumers but also by endemic diseases having to do with our civilized lifestyle (diabetes, obesity, or diseases affecting the nervous system and the digestive tract). In our time, we may still encounter many sceptical of the functional foods' beneficial effects, as these to take effect require regular consumption, and so their expected health benefits cannot be experienced instantaneously, but it may sometimes take months or even years.

In many countries of the developing world, the population is often grappling with protein deficiency due to the prevalence of low-protein-content plant foods. Protein deficiency can lead to growth retardation, oedema formation, and anaemia; if protein deficiency is coupled with lack of energy, malnutrition may cause the death of many infants and young children.

As a result of researches carried out in the past decades, today's subject-matter is not the protein needs in general but the specific quantitative requirements in terms of the essential amino acids. Researchers have also revealed that we must not only cover the deficiencies in limiting amino acids but we must also aim at achieving a balanced ratio of essential amino acids. Moreover,

we must pay close attention to obtaining an optimal ratio of essential/non-essential amino acids in our foods as well.

Once the industry-like production of amino acids had started, green light was given for amino acid supplementation, as a consequence of which a nearly optimal amino acid composition can be obtained, one of the methods leading up to producing protein of high biological value (*Rossel et al.*, 2016; *Albert et al.*, 2017a; *Prokisch et al.*, 2017a). Due to the widespread application of amino acid analysis, the amino acid composition of many food ingredients has become common knowledge, and by combining them we can again produce foods, such as bread, with optimal ingredients – yet another way of producing foods of high biological value.

The reasonable selection of food ingredients makes it possible the use of such proteins that can supplement the limiting amino acids of staple foods, such as flour, therefore ensuring an optimal composition for the developing organism. Recently, besides data on amino acid requirements, the optimal and minimal protein levels are also provided. In case of a sufficient protein level, the energy surplus will facilitate the energy-intensive process of protein synthesis, increase weight gain, and enhance benefits and protein utilization (*Rossel et al.*, 2016; *Csapó & Csapóné*, 2007; *Csapó et al.*, 2007).

# 2 Literature review

## 2.1 Enhancing the nutritional value of wheat flour and wheat-flour-based products

The protein needs of an adult person is 80–110 g/day depending on age and the exerted physical effort. In the case of a mixed diet, this amount of protein contains sufficient essential amino acids, but a one-sided diet may result in essential amino acid deficiency even if combined with an adequate protein consumption. Essential amino acids for an adult person include the following: isoleucine, leucine, lysine, methionine, phenylalanine, histidine, tryptophan, valine, and threonine (*Csapó & Csapóné*, 2004).

Proteins that contain all essential amino acids in a sufficient amount and in an adequate proportion are called complete proteins. Such are the proteins of meat, egg, and milk. Plant-based proteins, however – compared to the needs –, lack lysine, methionine, threonine, and tryptophan to varying degrees. Since there may occur certain abnormalities in the vital functions of those consuming small amounts of complete proteins, they supplement the largely plant-based foodstuffs with the missing essential amino acids. This supplementation would

be most needed in the case of cereal-based foodstuffs because the proteins of wheat and rye contain only small amounts of lysine, methionine, and threonine.

In Europe, people usually tend to opt for a supplementation with natural protein resources, for which the various soy-based products are the most suitable as soy protein contains a large amount of lysine and is stocked up with threonine above the average level. Its disadvantage is, however, the relatively low methionine and cystine content. Primarily, they used to fortify wheat-flour-based products – the efficiency of this protein supplementation is examined with the help of biological or chemical verification methods. Chemical methods can provide faster results, allowing us to determine the protein's amino acid composition and compare it to complete proteins by the calculation of chemical indices that give numerical information on the nutritional value of food proteins (*Csapó & Csapóné*, 2006; *Rossel et al.*, 2016).

The high lysine and threonine content of soya bean is an excellent supplement to the amino acid composition of the wheat flour, but an even better result can be obtained with a reasonably assorted protein supplementation, just as it happened in the case of the Update1 bread, where, besides soy protein, egg-white powder, gluten isolate, and dried yeast supplementation also took place. Due to protein supplementation, the carbohydrate content of wheat flour decreased by half, making this type of bread perfectly suited for the diet of diabetics and those who wish to lose weight.

## 2.2 The use of high-biological-value protein and of lysine in flour enrichment

The amount and ratio of amino acids in wheat is far from the optimal values necessary for the human organism, giving the nutritional value of wheat protein a score of approx. 53 on a scale of 100. Since there is a low proportion of lysine first of all, the high-lysine-content materials are the most suitable for the amino acid enrichment of wheat flour (potato (biological value: 73) and soya bean (biological value: 74–78)).

As the regular consumption of foodstuffs made from cereals is a widely used practice in our days, people have long been engaged in using trace elements (Se, Ca, Cu, Zn, Fe, P), vitamins (vitamin B family, vitamin E), proteins, and amino acids (tryptophan, lysine, threonine) in flour enrichment and fortification procedures.

Research on the clinical and dietetic application of L-lysine looks back to a history of 30–40 years. In 1976, *Titcomb* and *Juers* patented a type of bread with an amino acid composition corresponding to that of a complete protein.

In producing it, besides applying various protein supplementations, L-lysine hydrochloride was added to the flour until reaching a 0.1–0.5% proportion. *Mauron et al.* (1976) added to wheat flour the $\varepsilon$-amino acyl derivative of lysine, which would later create lysine during deacylation in the organism. In order to achieve a balanced amino acid composition, aside from acyl-lysine, an approx. 0.1% of L-lysine hydrochloride was also added to the flour. *El-Megged* and *Sands* (1990) elaborated a procedure during which the lyophilized sample of a lysine-producing lactic acid bacteria strain was added to the leaven, thus enriching the amino acid composition.

On the average, there was a 0.2–0.5% addition of lysine to the flour in order to increase the protein content. *Figueron et al.* (2005) added 0.5% of lysine to the flour, while *Muhammad et al.* (2012) reported a 0.2–0.3% enrichment. *Tajammal et al.* (2004) employed a 0.5% enrichment, yielding beneficial effects in children's development, increasing haemoglobin level in women, and elevating levels of transferrin in men. According to *Karcz* (2004), the consumption of 325 g of bread/day is recommended to satisfy 25% of people's daily needs of lysine.

*Wenhua et al.* (2004) conducted an experiment where the subjects consumed flour-based bread fortified with 0.3% of lysine, for a period of 3 months. They found that the consumption of lysine-enriched bread had positive effects on the immune system; although the haemoglobin level does not increase, the 0.3% lysine improves the immune system due to its effect on IgA, IgB, and IgE.

Several research results confirm the beneficial effects of lysine-enriched flours: *Anton et al.* (2008) and *Mora Aviles et al.* (2007) studied the effects of bean meal supplementation in tortilla, *Tyagi et al.* (2007) investigated the effects of mustard flour in rusk, while *Lindenmeier* and *Hofmann* (2004) looked into how a lysine derivative affects baking properties.

During a careful examination of the relevant literature, we did not come across any researcher who would have applied extracted soya bean meal, egg-white powder, and gluten or dried yeast in combination to produce bread with a low carbohydrate and high protein content, in which there would have been an outstanding essential amino acid ratio in the protein due to the extracted soya bean meal.

## 2.3 The physiological effects of the high lysine content of Update1 bread

It has been known for long that in the absence of L-lysine – the essential amino acid making up our proteins –, dietary calcium cannot get to the bones, and the synthesis of a number of proteins will also be inhibited. L-lysine plays a fundamental role in the formation of collagen that makes up the organic substance of the bone and skin, but its effect in strengthening the immune system and combatting viruses is also confirmed.

With respect to bone metabolism and the prevention of osteoporosis, the consumption of calcium, phosphorus, and vitamin D have been for long in the focus of literature as primary nutritional factors although vitamin C and lysine are also crucial in the formation of collagen making up the organic substance of the bones. Vitamin C takes part in the activation of vitamin D, on the one hand, and converts lysine, which takes part in building up the collagen substance of the bone, into a form suitable for the creation of procollagen, on the other hand. Research findings corroborate that, besides vitamin C, L-lysine is also a major contributor to the formation of a healthy bone structure (*Civitelli et al.*, 1992).

*Pauling* (1991) reported on the beneficial effects of lysine and vitamin C on the vascular system and on coronary artery diseases. *Rath* (2001) developed a product suitable for treating diseases related to the impairment of the extracellular matrix (atherosclerosis, cancer, infection, or other inflammatory diseases). The product contained lysine, proline, ascorbate, and their derivatives and synthetic analogues as well as vitamins, provitamins, and trace elements. In their study on L-lysine and the reaction of various carbohydrates, *Kitts and Hu* (2005) demonstrated that Maillard reaction products showed a significant antioxidant activity.

In summary, we can state that L-lysine intake in any form increases the biological value of lysine-deficient proteins, contributes to the optimal development of the young organism, and, besides its many therapeutic effects, can be effectively applied in fighting for the preservation of health.

## 2.4 The role of lysine in the formation of Maillard reaction products

Monosaccharides, reducing carbohydrates in general, with free amino group, react with each other under appropriate conditions, and by this reaction aroma compounds and brown-coloured pigments, so-termed melanoidins, are created.

This process is called the Maillard reaction. In the production of lysine-content bakery goods – as the $\varepsilon$-amino group of lysine is extremely sensitive to the Maillard reaction –, during the non-enzymatic browning reaction conducted at an appropriate temperature, antioxidants as well as colour and flavour substances are created, which contribute to the health-protective effect (*Csapó et al.*, 2006).

However, in planning the composition of the ingredients, we must also consider that the products resulting from high lysine content may spoil the product's palatability traits since the large amount of Maillard reaction products may come with unwanted flavour and colour effects (*Csapó&Csapóné*, 2006, 2007). We may say bread is a fortunate solution as temperature never rises above 100 °C on the inside, while in the bread-crust there is a proportionally low amount of lysine.

## 2.5 Humans' protein and amino acid requirements

The establishment of humans' protein requirements must take account of the protein loss in case of appropriate energy intake and a protein-free diet. This loss of endogenous protein is excreted from the body through urine, faecal matter, and perspiration, its amount also being enhanced by the wearing out of the skin, the growing hair and nails. On average, endogenous protein loss is approximately 0.34 g per body mass kilogram. Adult individuals' protein requirements can be satisfied with 0.75 g of good-quality protein per body mass kilogram, whereas in the case of infants, children, expectant and nursing mothers protein requirements are higher as the developing organism has to be provided with the necessary amount of protein for a proper growth and development (in the former two cases), but the protein needs of the foetus and of breast milk production must also be met (in the latter two cases). *Table 1* includes WHO data in relation to age on the safe amounts of protein intake when consuming milk or egg-white.

Naturally, the human body does not utilize protein itself but the amino acids found therein. For humans, histidine (depending on age), isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are all essential. Cystine, produced from methionine, and tyrosine, produced from phenylalanine, are semi-essential amino acids. In a healthy human body, intestinal microorganisms can synthesise histidine, wherefore amino acid is essential only for infants as the synthesised amount found in the intestines is perfectly sufficient for adult people's needs (*Csapó et al.*, 2007). *Table 2* includes the WHO recommendations on infants', young children's, and adults'

estimated amino acid requirements.

Table 1: Safe protein intake values (g/BMkg) for children according to the WHO recommendations

| Age (year) | Protein (g/BMkg) | Age (year) | Protein (g/BMkg) |
|---|---|---|---|
| Girls and boys together | | Girls | |
| 0.25–0.5 | 1.86 | 10–11 | 1.00 |
| 0.50–0.75 | 1.65 | 11–12 | 0.98 |
| 0.75–1.00 | 1.48 | 12–13 | 0.96 |
| 1.00–1.50 | 1.26 | 13–14 | 0.94 |
| 1.50–2.00 | 1.17 | 14–15 | 0.90 |
| 2–3 | 1.13 | Boys | |
| 3–4 | 1.09 | | |
| 4–5 | 1.06 | 10–11 | 0.99 |
| 5–6 | 1.02 | 11–12 | 0.98 |
| 6–7 | 1.01 | 12–13 | 1.00 |
| 7–8 | 1.01 | 13–14 | 0.97 |
| 8–9 | 1.01 | 14–15 | 0.96 |
| 9–10 | 0.99 | | |

BMkg = body mass kilogram

Table 2: Estimated amino acid requirements based on the WHO recommendations (mg/BMkg/day)

| Amino acid | Infants (3–4 months) | Young children (2 years) | School children (10–12 years) | Adults |
|---|---|---|---|---|
| Histidine | 28 | – | – | – |
| Isoleucine | 70 | 31 | 28–30 | 10.0 |
| Leucine | 161 | 73 | 44–45 | 14.0 |
| Lysine | 103 | 64 | 44–60 | 12.0 |
| Methionine + cystine | 58 | 27 | 22–27 | 13.0 |
| Phenylalanine + tyrosine | 125 | 69 | 22–27 | 14.0 |
| Threonine | 87 | 37 | 28–35 | 7.0 |
| Tryptophan | 17 | 12.5 | 3.3–4.0 | 3.5 |
| Valine | 93 | 38 | 25–33 | 10.0 |
| Total | 742 | 352 | 216–261 | 84.0 |

*Table 3* includes the optimal amount of amino acids in protein intake, based on the WHO recommendations, in comparison with the amino acid composition of wheat, soya, and egg protein. Nevertheless, data shown in this table may vary according to each individual's physical abilities, state of health, and due to several other reasons. Generally speaking, the average protein requirement is 0.6 g/BMkg, while the lowest amount of daily protein intake must not go below 0.45 g per BMkg.

Table 3: The optimal protein amino acid composition for infants, children, and adults in comparison with the amino acid composition of wheat, soya, and egg protein, based on the WHO recommendations

| Amino acid | Amino acid, mg/g protein | | | | | | |
|---|---|---|---|---|---|---|---|
| | Infants – average* | 2–5 years – children | 10–12 years – children | Adults | Wheat | Soya | Egg |
| Histidine | 26 | 19 | 19 | 16 | 23 | 32 | 22 |
| Isoleucine | 46 | 28 | 28 | 13 | 40 | 41 | 54 |
| Leucine | 93 | 66 | 44 | 19 | 71 | 77 | 86 |
| Lysine | 66 | 58 | 44 | 16 | 30 | 61 | 70 |
| Methionine + cystine | 42 | 25 | 22 | 17 | 41 | 31 | 57 |
| Phenylalanine + tyrosine | 72 | 63 | 22 | 19 | 78 | 88 | 93 |
| Threonine | 43 | 34 | 28 | 9 | 32 | 40 | 47 |
| Tryptophan | 17 | 11 | 9 | 5 | 12 | 16 | 17 |
| Valine | 55 | 35 | 25 | 13 | 48 | 47 | 66 |
| Total | | | | | | | |
| with histidine | 460 | 339 | 241 | 127 | 375 | 433 | 512 |
| without histidine | 434 | 320 | 222 | 111 | 352 | 401 | 490 |

*Estimated values based on the amino acid composition of mother's milk.

How can the main components of wheat protein meet these requirements? In terms of lysine, this is very difficult to accomplish since cereal proteins contain a relatively low amount of lysine. Among wheat proteins, albumins contain almost all proteinogenic amino acids. Their isoelectric point is about pH = 4–5. Globulins usually occur together with albumins, and their separation is possible based upon molecular mass. Globulins dissolve in dilute saline and some of them also in distilled water. These latter are called pseudo-globulins in contrast to euglobulins, which dissolve in dilute saline. A high concentration of proline and glutamic acid is peculiar to prolamins – they do not contain any lysine. Gliadin and glutenin together form the complex protein of gluten, which is a key protein in wheat noodle production. Glutenins are typical plant proteins found in seeds. Arginine, proline, and glutamic acid

are predominantly present in their amino acid composition; the best known variant is the glutenin found in wheat. Thus, the amount of lysine is low in cereal crops (2–4%), which is a limiting factor for the biological value of many proteins of plant origin.

Under normal nutritional conditions, there is no risk of lysine overnutrition. If, however, an amount of lysine in excess of actual needs gets into the system, it will be utilized as energy (*Csapó* and *Csapóné*, 2007).

## 3 Aims of the experiments

The aim of our research is to produce a type of bread as a functional food, fortified with lysine and other essential amino acids, which helps in satisfying the organism's optimal essential amino acid needs and has beneficial effects in the treatment of osteoporosis and other diseases of the circulatory system. We expect that adding a right dosage of extracted soya bean meal, egg-white powder, gluten, and dried yeast to wheat flour will increase the amount of essential (and, in the case of wheat flour, limiting) lysine as well as the biological value of wheat protein, whereas that adding an amount higher than necessary for increasing the biological value will result in such a functional, health-protective, and health-preserving product that will help prevent diseases caused by amino acid deficiency, such as weakness of the bones or osteoporosis.

As during the baking process bread-crumb temperature does not exceed 100 °C, we were hoping for a practically unchanged lysine content inside the bread, while in the bread-crust and directly underneath of it lysine would be transformed in significant quantities into colour and flavour substances (Maillard reaction) as well as antioxidants. We were also confident that the application of egg-white powder and dried yeast would let us introduce those essential amino acids into the bread that can be found in relatively small amounts in soya bean (methionine, cystine). The sole purpose of applying gluten was to increase protein content and decrease carbohydrate content, while the added bamboo fibre also served to decrease the starch content and increase fibre content.

In summary, the point of our solution is to produce a type of bread with a complete amino acid composition, high essential amino acid content, increased protein and fibre content, and reduced starch content. Therefore, we mixed together wheat flour of 12–13% protein content with egg-white powder of 83% protein content, gluten of 79.4% protein content, extracted soya bean flour of 49.3% protein content, dried yeast of 16% protein content, and the practically

protein-free bamboo fibre so that we could obtain a flour of high biological value suitable for the production of functional foods and also having health-protective and health-preserving effects. In this article, we report on our research results regarding the Update1 bread.

# 4 Materials and methods

## 4.1 Composition of the basic ingredients for bread and the baking process

In planning the composition of the ingredients, we had to consider the amino acid content of the ingredients and of the end-product, the relative proportion of the amino acids, and the balanced volume of non-essential amino acids next to the essential amino acids. We had to pay attention and avoid at all costs the unwanted flavour and colour effects of the large amount of Maillard reaction products due to the increased lysine content.

The raw mixture was composed of wheat flour of 12–13% protein content, egg-white powder of 83.0% protein content, gluten of 79.4% protein content, extracted soya bean flour of 49.3% protein content, dried yeast of 16% protein content, and the practically protein-free bamboo fibre. The percentage composition of the mixture is protected by patent, and therefore we cannot disclose it. Following the baking process, during which we applied special temperature and timing combinations, we examined all attributes of the breads that are also applied in practice upon the evaluation of breads. Throughout the pilot production phase – depending on the nature of the product –, the dough was leavened, shaped, and then baked in an automatic bread-baking machine. With the preliminary results in hand, we adjusted baking temperature and time as required, depending on whether we intended to produce a bread with higher lysine or higher antioxidant content.

## 4.2 Chemical-analytical investigations

### 4.2.1 Determination of total protein and amino acid composition

Prior to analytical investigations, we let the bread dry at room temperature, milled it to the fineness of flour, and used this homogeneous material in our examination. The total protein content of the breads was determined with the help of VelpScientifica UDK 159, an apparatus operating on the principle of the Kjedahl method. The protein was hydrolysed with 6M HCl at 110 °C for

24 hrs, and the determination of amino acids was carried out with the high-performance liquid chromatography method with orto-phthalaldehyde (OPA)-2-mercaptoethanol pre-column derivatization. Apparatus used: Varian Pro Star; method applied: fluorescence detection ($\lambda$ex = 340 nm, $\lambda$em = 455 nm); column: Pursuit C18 5$\mu$m, 250 × 4.6 mm; gradient elution: A: 100 mmol/l acetate puffer (pH = 6.95) 925 ml, methanol 50 ml, tetrahydrofuran 25 ml, B: methanol 975 ml, tetrahydrofuran 25 ml; flow rate: 0.8 ml/min; gradient program for the analysis: 0–8 min; A component 100%, B component 0%, 8–11 min: 90–10, 11–16 min: 75–25, 16–20 min: 60–40, 20–24 min: 60–40, 24–27 min: 40–60, 27–32 min: 0–100, 32–33 min: 0–100, 33–47 min: 100–0. Under the conditions employed, there was a fairly clear separation of the amino acids, while quantification was not disturbed by any circumstances.

In the determination of tryptophan, the protein was hydrolysed with 4M sodium hydroxide at 110 °C for 24 hrs, and then we determined tryptophan content using once again the high-performance liquid chromatography method.

### 4.2.2 Determination of HMF

In order to estimate the amount of Maillard reaction products, high-performance liquid chromatography was used to determine the hydroxymethylfurfural (HMF) concentration of the bread. For the measurement of HMF, samples taken from the different types of bread were ground in electric mill to 0.75 mm sieve size. An amount of 1 g was taken from each sample, to which we added 9 ml of de-ionized water, and then we had it mixed with a Vortex tube mixer. Following this, with a view to eliminate any interfering substances, 0.5 ml of Carrez I and Carrez II solution was added to the extract, and then centrifuged for 10 minutes at 5,000 rpm. The supernatant was removed and filtered through 0.45 $\mu$m filtration hoods, and 20 $\mu$l of the obtained solution was injected into the Varian Pro Star HPLC apparatus. Isocratic elution method was used to measure HMF, where the mobile phase was a 20:80% solution of 5% methanol-acetic acid, the stationary phase was Pursuit C18 column (250 × 45 mm), the rate flow was 1ml/min, and UV detection was performed at a wavelength of 285 nm.

### 4.2.3 Calculating the biological value of the protein

Determination of protein quality starts with determining protein content, followed by determining the amino acid composition of the protein. Data on amino acid composition let us determine the limiting amino acid of the pro-

tein as compared to the amino acid composition of the reference protein made according to the FAO/WHO recommendations. The amino acid occurring in the lowest percentage is the limiting amino acid, whose numerical value is the chemical index (CS). Besides CS, other indices can also be calculated from the amino acid composition if we consider the rest of the essential and non-essential amino acids as well.

Nowadays, during the determination of biological value in human terms, many tend to use the Morup and Olesen (1976) biological value calculation method, who, based on their experiments carried out with young male university students, experienced the highest rate of nitrogen retention when the experimental subjects were consuming a mixture made up of 66% potato and 34% whole egg, which in our country is mostly similar to layered potato casserole of a very good quality, prepared without sausages. They elaborated the following equation for the calculation of biological value:

$$\text{Biological value} = 10^{2.15} \cdot q_{Lys}^{0.41} \cdot q_{Arom}^{0.60} \cdot q_{Sulf}^{0.77} \cdot q_{Thr}^{2.41} \cdot q_{Trp}^{0.21},$$

where:

$q = a/a_{ref}$,

$a$ = $a$ particular essential amino acid/all essential amino acids in the protein under examination,

$a_{ref}$ = $a$ particular essential amino acid/all essential amino acids in the reference protein,

$Arom$ = aromatic amino acids (Tyr, Phe),

$Sulf$ = sulphur-containing amino acids (Cys, Met).

According to this, the biological value of the reference protein is $10^{2.15} \cdot 1 \cdot 1 \cdot 1 \cdot 1 \cdot 1$, that is = 141.25. When calculating the biological value, aromatic amino acids (phenylalanine + tyrosine) and sulphur-containing amino acids (methionine + cystine) were evaluated together besides lysine, threonine, and tryptophan.Among amino acids, tryptophan figures on the 0.21 power, and thus it has the lowest influence on biological values, while threonine figures on the 2.41 power, having the highest influence on biological value. As for the rest of the amino acids, this index varies between 0.41 and 0.77, thus exerting by and large a similar degree of influence on biological value.

### 4.3 Sensory analysis

During the sensory analysis of the breads, we evaluated their shape (typical of bread (loaf shape), regular, proportionately convex, not deformed), crust (typ-

ical of the particular type of bread; shiny, smooth or cracked; scattered and/or sliced; not split all the way, not sooty/soiled/burned/saturated/damaged), internal content (baked thoroughly; does not separate from the crust; the colour is typical of the flour used; the substance is consistent, flexible, and free of lumps; not lardy, sticky, crumbly, or falling apart; does not contain foreign substances), as well as taste and aroma (aromatic – typical of the particular type of bread, not having strange taste or odour). Sensorial analyses were carried out as prescribed by Codex Alimentarius (2004).

### 4.4 Statistical evaluation of the data

We used Microsoft Excel 2010 software package and one-way analysis of variance to perform the statistical evaluation of the data.

## 5 Results and discussion

### 5.1 Protein content and amino acid composition of bread ingredients

To determine the total protein content, the samples taken from the different types of bread were ground in electric mill to 0.75 mm sieve size and passed through a sieve with such hole size, and then an amount of 2 g from the wheat flour and – due to the higher protein content – 1 g from the other ingredients were digested. Digestion and distillation of ammonia were equally repeated three times.

Prior to determining the essential amino acids, the proteins of wheat flour and of the ingredients were hydrolysed with 6M HCl at 110 °C for 24 hrs, and then, after proper dilution and derivatization, 20 $\mu$l of hydrolysate was injected into the high-performance liquid chromatography. In the determination of tryptophan, the protein was hydrolysed with 4M sodium hydroxide at 110 °C for 24 hrs, and then we determined tryptophan content using once again the high-performance liquid chromatography method.

The protein content of wheat and the wheat flour produced from it varies between 11% and 14% depending on the species and growing conditions. Among its protein fractions, albumins are water soluble, globulins are soluble in 0.4 mol/l NaCl saline, prolamins are soluble in 70% aqueous ethanol solution, and glutenins form the fraction remaining from the flour after the sequential extraction. Upon dissolving the glutenins in 60% aqueous solution of 1-propanol, the high-molecular-weight (HMW) subfraction precipitates, whereas the low-

molecular-weight (LMW) subfraction remains in the solution. Enzymes can be ranked among albumin and globulin fractions, while prolamins and glutelins form the category of storage proteins. In wheat, the albumin fraction amounts to 14.7% of the total protein, the globulin 7%, the prolamin 32.6%, and the glutelin 45.7%. In the case of wheat, the albumin is termed leucosine, globulin is edestin, prolamin is gliadin, and glutelin is glutenin.

Low lysine and methionine content is characteristic of the amino acid composition of cereal crops – wheat contains an especially low amount of methionine.100 g of wheat (with 13.2% protein content) contains 0.73 g aspartic acid, 0.42% threonine, 0.69% serine, 3.75% glutamic acid, 1.36% proline, 0.55% glycine, 0.53% alanine, 0.32% cystine, 0.63% valine, 0.22% methionine, 0.53% isoleucine, 0.94% leucine, 0.42% tyrosine, 0.61% phenylalanine, 0.30% histidine, 0.40% lysine, 0,65% arginine, and 0.16% tryptophan.

Wheat gluten (with 79.4% protein content) contains 4.4% aspartic acid, 2.8% threonine, 4.8% serine, 31.9% glutamic acid, 12.1% proline, 3.5% glycine, 2.4% alanine, 1.8% cystine, 4.6% valine, 1.7% methionine, 4.1% isoleucine, 7.2% leucine, 3.1% tyrosine, 4.7% phenylalanine, 2.3% lysine, 1.8% histidine, 3.9% arginine, and 1.1% tryptophan. Its amino acid composition predominantly includes glutamic acid and proline, these two non-essential amino acids amounting to 44.0% of the total protein content.

The other main ingredient of Update1 flour is extracted soya bean meal with 49.3% protein content. 60% of the mass of the soya bean is made up of protein and oil, approx. 35% carbohydrates, and the remaining 5% is ash. Most part of the protein is heat-resistant, which is a significant factor since procedures aiming at reducing or eliminating trypsin inhibitor activity involve heat treatment, which does not damage soya protein provided a proper technology is applied. Carbohydrates of soya bean include saccharose, raffinose, and stachyose, indigestible to humans but still useful for their water-binding properties as they protect seeds from desiccation. Its insoluble carbohydrates include cellulose, hemicellulose, and pectin, also indigestible to humans but with significant beneficial physiological effects. The oil content of soya bean will not be discussed here as the production of the Update bread made use of extracted soya bean flour, which contains only traces of oil.

Its mineral content is approx. 5% including a significant amount of macroelements (potassium, calcium, magnesium, and phosphorus) and microelements (mostly zinc and iron). It contains a considerable amount of B-group vitamins (pantothenic acid, pyridoxine, folic acid, thiamine, niacin, and riboflavin) as well as vitamins E and K. Its vitamin C content is negligible.

Its protein content ranges between 36% and 37%, while the protein content

of the extracted soya bean meal can reach 50%. 100 g of mature, air-dry soya bean contains 5.10 g aspartic acid, 1.77 g threonine, 2.36 g serine, 7.87 g glutamic acid, 2.38 g proline, 1.88 g glycine, 1.93 g alanine, 0.66 g cystine, 2.03 g valine, 0.55 g methionine, 1.97 g isoleucine, 3.31 g leucine, 1.54 g tyrosine, 2.12 g phenylalanine, 2.71 g lysine, 1.10 g histidine, 3.15 g arginine, and 0.59 g tryptophan. If we multiply the values by two, we will get the amino acid content of the soya protein.

The third main component of Update1 bread is egg-white powder. Except for fibre, egg contains almost all valuable nutrients. The biological value of its protein is perhaps the highest among all food proteins, which is why it may be considered a protein that serves as a reference base in the calculation of other proteins' biological value. Its yolk contains large amounts of fats and vitamins; this way, the egg white and egg yolk together can meet human requirements almost optimally. Therefore, if properly prepared and without changing its composition, it can be considered a functional food.In summary, egg is rich in nutrient content, being one of the most valuable naturally created foods. It has great versatility in its industrial and home use, and besides the hen's egg our diet also includes the eggs of duck, goose, lapwing, seagull, and quail. Egg white is a 10% aqueous solution of proteins, which, aside from proteins, also contains 0.03% lipids and 1% carbohydrates. The pH of a fresh egg is between 7.6 and 7.9, which can go up to 9.7 during storage time as the soluble $CO_2$ diffuses from the egg through the eggshell. pH increase is a time- and temperature-dependent procedure. At 35 °C in 21 days, pH increases to 9.4. Albumin proteins with a biological activity provide protection against microbial degradation. In this manner, for instance, cleaving the peptidoglycans that form the bacterial cell walls, lysozyme enzyme kills the bacteria, while enzyme inhibitors (ovomucoid, ovoinhibitor) and substances forming complexes with the coenzymes (e.g. flavoprotein, avidin) also play the same role.

100 g of egg white contains 6.0 g aspartic acid, 3.4 g threonine, 6.0 g serine, 10.9 g glutamic acid, 2.9 g proline, 2.9 glycine, 5.5 g alanine, 1.9 g cystine, 6.0 g valine, 3.0 g methionine, 5.0 g isoleucine, 6.8 g leucine, 3.2 g tyrosine, 4.9 g phenylalanine, 4.6 g lysine, 1.7 g histidine, 4.5 g arginine, and 1.2 g tryptophan.

The dried yeast with 16% protein content used in bread baking is so small in quantity compared to the above-listed components that it has no effect whatsoever on the amino acid composition of the flour and that of the bread produced from it.

Based on measurement data, we can establish that Update1 flour is a flour mixture of high protein and fibre content and reduced carbohydrate content,

produced from natural ingredients, being sufficient of itself to be used in the preparation of various products with reduced carbohydrate content (e.g. breads, bakery products, desserts, pastries, muffins, etc.). It might be a convenient solution for those who do not like to experiment with the proportions. It contains fine-ground wheat flour, defatted soya bean flour, wheat gluten, dried yeast, bamboo fibre, and soya protein isolate. According to official data, its caloric value is 1364 kJ/100 g, its fat content 1.4%, of which the saturated fatty acid less than 0.5%, its carbohydrate content 35.4%, of which sugar 2.8%, starch 32.6%, fibre 14.4%, protein 35.0%, and salt 0.125%. Overall, it can be said this is a natural food ingredient with a protein content almost three times the fine-ground flour and a carbohydrate content decreased by half.

### 5.2 Total protein content of the bread

To determine the total protein content, the samples taken from the different types of bread were ground in electric mill to 0.75 mm sieve size, and an amount of 2 g from the regular bread and – due to the higher protein content – 1 g from the Update1 bread were digested. Digestion and distillation of ammonia were equally repeated three times. We measured the following amounts of protein content: control bread – 12.4±0.18%, Update1 bread – 35,2±0,26%. Results immediately make it clear that due to the added extracted soya bean flour, egg-white powder, and gluten, the protein content of the Update1 bread has increased 2.86 times compared to the control bread. This increase was an expected outcome as all of the additives had a higher protein content than the wheat flour.

### 5.3 Essential amino acid composition of the control and the Update1 bread

Prior to determining the essential amino acids, the proteins of the breads were hydrolysed with 6M HCl at 110 °C for 24 hrs, and then, after proper dilution and derivatization, 20 $\mu$l of hydrolysate was injected into the high-performance liquid chromatography. In the determination of tryptophan, protein was hydrolysed with 4M sodium hydroxide at 110 °C for 24 hrs, and then we determined tryptophan content using once again the high-performance liquid chromatography method. *Table 4* includes the essential amino acid content of wheat flour, extracted soya bean meal, egg-white powder, gluten, control bread, and the Update1 bread as well as the biological value of the protein.

Table 4: Essential amino acid content of wheat flour, extracted soya bean meal, egg-white powder, gluten, control bread, and the Update1 bread as well as the biological value of the protein

| Amino acid | Amino acid, mg/g protein | | | | | | |
|---|---|---|---|---|---|---|---|
| | Wheat flour | Extracted soy bean meal | Egg-white powder | Gluten | Control bread | Update11 | Update12 |
| Histidine | 23 | 32 | 22 | 18 | 21 | 27 | 26 |
| Isoleucine | 40 | 41 | 54 | 41 | 40 | 44 | 43 |
| Leucine | 71 | 77 | 86 | 72 | 71 | 78 | 79 |
| Lysine | 30 | 61 | 70 | 23 | 26 | 46 | 47 |
| Methionine + cystine | 41 | 31 | 57 | 35 | 38 | 41 | 41 |
| Phenylalanine + tyrosine | 78 | 88 | 93 | 78 | 77 | 87 | 86 |
| Threonine | 32 | 40 | 47 | 28 | 30 | 38 | 39 |
| Tryptophan | 12 | 16 | 17 | 11 | 11 | 15 | 14 |
| Valine | 48 | 47 | 66 | 46 | 49 | 52 | 51 |
| Total | | | | | | | |
| with histidine | 375 | 433 | 512 | 352 | 363 | 428 | 426 |
| without histidine | 352 | 401 | 490 | 334 | 342 | 401 | 400 |
| Biological value* | 53 | 75 | 98 | 47 | 51 | 74.8 | 74.7 |

* Calculated according to Morup and Olesen's (1976) method.

As it can be seen from the table, wheat flour and gluten contain the smallest amounts of essential amino acids among the applied ingredients, yielding therefore the lowest biological value for the protein of wheat flour (53) and gluten (47). The high lysine and threonine content of the extracted soya bean meal is in line with expectations, but its sulphur amino acid content is rather moderate, and so the biological value of the protein is 75. Due to its outstanding essential amino acid content, egg white disposes of the highest biological value (98), and thanks to its high methionine and cystine content it serves as an excellent supplement to the low levels of sulphur amino acids in the soya and wheat protein as well as in gluten. The high lysine and, especially, threonine content of egg white also contributes to the high biological value of the Update1 bread for which it serves as a supplement. Owing to its low lysine, threonine, and sulphur amino acid content, gluten has the lowest biological value.

As a consequence of the amino acid composition of the additives used during dough preparation and due to the baking processes, the control bread has practically the same level of amino acid content as wheat flour, but because of a minimal loss of lysine, methionine, and cystine, its biological value is slightly lower.

The table shows data on the Update1 bread from the two different batches. Since data practically coincide, the two measurement results can be evaluated together. Due to the high histidine content of the extracted soya bean meal, the histidine content of the protein in the Update1 bread is slightly higher (27 mg/g) than that of the control bread. It has a slightly higher isoleucine content, which is a consequence of the egg white's higher isoleucine content. The leucine content is again a higher one as both soya protein and egg white have a higher leucine content than wheat protein. It has an approx. 80% higher lysine content than the control bread owing to the higher lysine content of the soya bean and egg white. Despite supplementation, its sulphur amino acid content (methionine, cystine) is not higher than that of the control bread due to the low sulphur amino acid content of the soya bean, which is, however, compensated by the sulphur amino acid content of the egg white, being well above the required level. On the whole, the sulphur amino acid content of the protein in the control bread and the Update1 bread may be considered identical.

The protein of the Update1 bread has a more than 10% higher aromatic amino acid (tyrosine, phenylalanine) content, while there is an even bigger difference, of almost 30%, in the threonine content. This 30% difference can also be observed in the tryptophan content, but the valine content of the

two proteins shows only a slight difference in favour of the Update1 protein. (Regarding all amino acids, evaluation refers to the protein and the amount of amino acids. As the Update1 bread contains protein in an amount of almost three times the control bread, the sulphur amino acids are also present in the Update1 bread in a concentration almost three times higher, while in regard to the rest of the amino acids this difference can be even four- of fivefold.)

Using Morup and Olesen's (1976) method in our calculation, the biological values are as follows: gluten – 47, wheat flour – 53, soya bean meal – 75, and egg-white powder – 98. The biological value of the control bread was measured at 61 and that of the Update1 bread at 75. All in all, the wheat protein's biological value of 53 could be raised to 75 with the help of extracted soya bean meal, egg-white powder, and gluten supplementation, which is a more than 40% increase. (In all cases discussed, data refer to the protein.)

Comparing the protein content of the Update1 bread and the amino acid composition of the protein with data shown in tables 1–3, we may state that 3–4 slices of Update1 bread can cover most part (60–70%) of an adult's daily essential amino acid requirements.

### 5.4 Amount of the hydroxymethylfurfural

Taking an average of five measurements, the hydroxymethylfurfural (HMF) content of the control bread was $0.98 \pm 0.05$ and that of the Update1 bread $4.56 \pm 0.34$ mg/kg. The examination of data using one-way variance analysis suggested significant differences, meaning that the hydroxymethylfurfural content increased in the breads consequent upon the addition of soya protein and egg white, which can contribute to imparting a flavour to the Update1 bread and increasing the volume of the compound with antioxidant properties, but, to a slight, almost insignificant degree, it can also reduce the amount of the available lysine.

### 5.5 Sensory analysis results

Based on the analyses carried out as prescribed by Codex Alimentarius (2004), only minimal differences could be observed concerning the following properties of the Update1 and the control bread: shape (typical of bread (loaf shape), regular, proportionately convex, not deformed), crust properties (typical of the particular type of bread; shiny, smooth or cracked; scattered and/or sliced; not split all the way, not sooty/soiled/burned/saturated/damaged), internal content (baked thoroughly; does not separate from the crust; the colour is

typical of the flour used; the substance is consistent, flexible, and free of lumps; not lardy, sticky, crumbly, or falling apart; does not contain foreign substances), and taste and aroma (aromatic – typical of the particular type of bread, not having strange taste or odour). Although in conformity with the higher lysine and other amino acid content the taste and aroma of the Update1 bread differs from those of the traditional bread, this difference in taste and aroma is not a disagreeable one, and one can get used to it in a matter of days. A significant difference, however, was observed in the colour of the crust and the internal content, according to which the internal content of the Update1 bread had a darker shade, while the crust could come in hues that go all the way to dark brown, which is a consequence of the higher lysine content and the Maillard reaction during the baking process.

## 6 Conclusions

We determined the composition of ingredients used in the production of the Update1 bread, mixed them in appropriate proportions, and produced a type of flour with a composition whose caloric value was 1364 kJ/100 g, its fat content 1.4%, of which the saturated fatty acid less than 0.5%, its carbohydrate content 35.4%, of which sugar 2.8%, starch 32.6%, fibre 14.4%, protein 35.0%, and salt 0.125%. This mixture is a natural food ingredient with a protein content almost three times the fine-ground flour and a carbohydrate content decreased by half.

Wheat flour and gluten contained the smallest amounts of essential amino acids among the applied ingredients, yielding therefore the lowest biological value for the protein of wheat flour (53) and gluten (47). The high lysine and threonine content of the extracted soya bean meal is in line with expectations, but its sulphur amino acid content is rather moderate, and so the biological value of the protein is 75. Due to its outstanding essential amino acid content, egg white disposes of the highest biological value (98), and thanks to its high methionine and cystine content, it serves as an excellent supplement to the low levels of sulphur amino acids in the soya and wheat protein as well as in gluten.

Comparing the amino acid composition of the protein in the control bread and the Update1 bread, we could establish that except for methionine and cystine, the protein of the Update1 bread contains substantially more essential amino acids, as a consequence of which the biological value of the control bread protein was 61 and that of the Update1 bread 75. If we also take into account

that the Update1 bread contains protein almost three times the control bread, which difference is therefore threefold in terms of sulphur amino acids and four- to fivefold regarding the rest of the amino acids, we may draw the conclusion that 2–3 slices of Update1 bread can cover 60–70% of an adult's daily essential amino acid requirements.

Based on the sensory analyses, in conformity with the higher lysine and other amino acid content, the taste and aroma of the Update1 bread, the colour and substance of the crust and internal content differ from those of the regular bread due to the applied supplements with a higher protein content.

In conclusion, we can say that the Update1 bread is a functional food with an outstanding biological value, a good taste, high protein and amino acid content, containing proteins almost three times and essential amino acids three to five times the traditional bread, and thus able to meet humans' amino acid requirements with a great efficiency.

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# Toxicity of selenium, application of selenium in fertilizers, selenium treatment of seeds, and selenium in edible parts of plants

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**Abstract.** Agronomic biofortification is one of the approaches which have been successfully adopted for improving the nutritional content of plant-based foods and is mainly focused on optimizing the application of mineral fertilizers and/or the improvement of the solubilization and mobilization of mineral elements in the soil. In general, mineral elements with a good dynamism in the soil and in the plant are good candidates for a prosperous agronomic biofortification. Selenium deficiency occurs in areas where soil Se is low, including parts of Europe, China, North America, Australia, New Zealand, and Southern Africa. Selenium toxicity occurs in areas where soil Se is naturally high, including areas of China, India, and the United States. Toxicity from naturally occurring Se may be intensified by irrigation of seleniferous soils, mining, and use of Se-rich fossil fuels. Then, management practices benefit from a thorough understanding of the mechanisms of plant Se uptake and the fate of Se in different plant species.

**Keywords and phrases:** selenium, toxicity of selenium, selenium–sulphur interrelation, selenium in plants, selenium in fertilizers

# 1 Toxicity of selenium and its tolerance in plants

Accumulation of Se in the tissues of plants lead to symptoms of toxicity, such as stunting, chlorosis, and fading of leaves, between 2 mg $kg^{-1}$ in non-accumulators, such as rice, and 330 mg $kg^{-1}$ in white clover (*Mikkelsen et al.*, 1989), to several thousands of mg $kg^{-1}$ in the accumulator *Astragalus bisulcatus* (*Shrift*, 1969). There are some factors that help identify the susceptibility of a certain plant to toxicity, such as Se concentrations, levels of sulphate in the soil, the stage of growth, and the chemical form of the concentrated Se. It has been observed that both selenite ($SeO_3^{2-}$) and selenate ($SeO_4^{2-}$) are major forms of toxic to non-accumulators because they are readily absorbed and assimilated by plants (*Wu et al.*, 1988).

The interpolation of seleno-amino acids, selenocysteine, and selenomethionine into proteins instead of cysteine and methionine and then the changes in the tertiary structure, resulting from differences in size and ionization properties between S and Se atoms, may have a negative effect on the catalytic activity of certain important proteins and may induce the major mechanism of Se toxicity (*Brown & Shrift*, 1982). It has also been found that Se induces toxicity in plants by interfering with chlorophyll combination (*Padmaja et al.*, 1989) as well as with nitrate accumulation (*Aslam et al.*, 1990). There is also proof that Se can interfere with the production of glutathione, and thus decrease a plant's defence against hydroxyl radicals and oxidative stress (*Bosma et al.*, 1991). It appears that there is a high tolerance of accumulators regarding Se levels that would result in toxicity in non-accumulators because of the decrease of intracellular concentrations of selenocysteine and selenomethionine, hence preventing their interpolation into proteins. This is brought about by turning the Se into non-protein seleno-amino acids, such as selenocystathionine, or into the dipeptide $\gamma$-glutamyl-seleno-methyl-selenocysteine (*Nigam et al.*, 1969). There is some proof that this may, to some amount, be achieved by the compartmentation of the element in the form of selenate or, maybe, as non-protein seleno-amino acids, in vacuoles (*Terry et al.*, 2000), as suggested by *Reilly* (2006).

It has been reported that toxic Se concentrations in non-accumulator plants result in a 10% decrease of yield, without any visible symptoms (Se contents between of 2 and 330 mg $kg^{-1}$ in rice and white clover resp.). In accumulator plants, Se concentration may reach 4,000 mg $kg^{-1}$, without any negative effects (*Kabata-Pendias*, 2011). Tolerance mechanisms include processes of exclusion of active Se amino acids, thus preventing their incorporation into proteins and the damaging effects on plant functions (*Terry et al.*, 2000). The deprivation

of Se from proteins in accumulator plants is the basis for their tolerance to Se. Generally, food crops have a low Se tolerance; however, most other plants may accumulate amounts of Se that are toxic for humans and animals. In non-tolerant plant species, a surplus of Se may destroy germination and growth and cause chlorosis and black spots on leaves. Increased Se levels in plants repress their concentrations of N, P, and S, just as different amino acids do; thus, high Se concentrations inhibit the absorption of metals, mainly Mn, Zn, Cu, and Cd. These connections are dependent on the ratio between the elements, and therefore it is possible for high Se levels to have stimulating effects on the uptake of some trace elements. The application of N, P, and S is known to help in detoxifying Se, which is perhaps a result either of suppressing Se uptake by the roots or of establishing a beneficial ratio of Se with these elements, as suggested by *Kabata-Pendias* (2011).

It has been reported that when Se-sensitive plants are subjected to high levels of Se in the soil-root medium, they may show various symptoms such as short growth, chlorosis, withering, drying of leaves, and premature death of the entire plant (*Mikkelsen et al.*, 1989). In general, the threshold range in non-accumulator plants varies with plant age and S supply as younger plants can be more sensitive to toxicity, while tolerance to Se toxicity reaches higher levels with increasing sulphate supply (*Brown* and *Shrift*, 1982). The threshold toxic content in non-accumulator plants also depends on the form of Se applied and with selenate and selenite being the major toxic forms for plants. This may be linked to both these forms of Se readily absorbed and translocated in plants and assimilated in the inorganic forms (*de Filippis*, 2010).

We can conclude a number of possible modes of tolerance to toxic compounds, which are described by *Pilon-Smits* (2005) and may involve any of the six mechanisms – these include differences in adsorption, conjugation, sequestration, enzymatic modification, enzymatic degradation, and volatilization. Tolerance in Se-accumulator plants is apparently due to a number of mechanisms such as: 1) Adsorption or transportation: decrease in excessively high concentrations of Se being transported into the cells of leaves. 2) Sequestration or enzymatic modification: accumulation of Se in seleno-amino acids, but these seleno-amino acids are not incorporated into normal protein synthesis. 3) Sequestration: compartmentation of Se as selenate in the vacuole and away from more sensitive cytoplasmic reactions. 4) Enzymatic modification: increase ATP sulphurylase and SeCys methyltransferase activities to decrease inorganic Se to organic forms of Se, although other enzymes and reactions are also required. 5) Conjugation: conjugation with glutathione (GSH) and an increase in antioxidation protective reactions. Conjugation with Se-binding

proteins and polypeptides decreases inorganic Se content. 6) Volatilization: increase volatilization of mainly organic forms of Se out of plant cells and tissues (*de Filippis*, 2010).

It is a well-known fact that plant species vary in their ability to accumulate Se and most plants have less than 25 mg Se $kg^{-1}$ dry matter and are called non-accumulators. Such plants are unable of tolerate high Se in the surrounding, and Se toxicity occurs under about 10–100 mg Se $kg^{-1}$ of dry matter, although the exact value depends upon the selenate:sulphate ratio in the rhizosphere solution (*White et al.*, 2004). These plants tolerate low Se concentrations in the rhizosphere by limiting Se uptake and movement to the shoot (*Wu et al.*, 1988). Different plant species can grow sufficiently in both seliniferous and non-seliniferous soils, and they may contain more than 1,000 mg Se $kg^{-1}$ dry matter without consequence (*White et al.*, 2004).

Therefore, we may conclude that both selenite ($SeO_3^{2-}$) and selenate ($SeO_4^{2-}$) are main forms that are toxic to non-accumulators because they are readily absorbed and assimilated by plants (*Domokos-Szabolcsy et al.*, 2011). Additional Se levels in plants repress their concentrations of N, P, and S, like different amino acids, thus high Se concentrations prevent the absorption of metals, originally Mn, Zn, Cu, and Cd. These communications are dependent on the ratio between the elements. The application of N, P, and S is believed to help in detoxifying Se, which is perhaps a result either of suppressing Se uptake by the roots or of establishing a beneficial ratio of Se with these elements.

## 2 Selenium–sulphur interrelation

S and Se have received little attention so far with respect to biotechnology-based crop improvement, at least when compared with nitrogen or phosphorus. In pursuit of a higher yield, better nutritional value, and quality in combination with sustainable plant management, various biotechnological approaches have tried to modify crop plants in recent years (*Khan & Hell*, 2008). Plant nutritional aspects may be the major reason for this lack of interest of S and Se. Moreover, S is the least necessary one among the six macronutrients and is usually available in sufficient amounts in soils of arable land. Its mineral fertilization is relatively affordable, often combined with chemically decreased nitrogen (ammonium sulphate), and even S contaminations of nitrogen and phosphate mineral fertilizers may be sufficient to support crop growth in some cases (*Pasricha & Abrol*, 2003). On the other hand, Se still has not been pointed out as a necessary nutrient for plants (not regarding algae), and it

just plays the role of a potentially deleterious component in small agricultural areas with great selenate content in the soil. However, many decreased Se compounds, such as methionine and different vitamins (*Hell*, 1997), are necessary in the human diet as is selenide in a steadily increasing number of specialized enzymatic actions (*Sors et al.*, 2005b).

The close relationship between Se and S metabolism in plants may be due to the physical and chemical similarities of Se and S. Both S and Se are part of Group 16 in the periodic table, the most common valence states of S and Se being $-2, 0, +2, +4, +6$, with Se occurring as $Se^{2-}$ (selenite), Se0 (elemental selenium), $Se_2O_3^{2-}$ (thioselenate), $SeO_3^{2-}$ (selenite), and $SeO_4^{2-}$ (selenate) respectively. The predominant forms of S and Se available for plants are $SO_4^{2-}$, $SeO_4^{2-}$, and $SeO_3^{2-}$ (*Sors et al.*, 2005a). These elements have some chemical differences, from which one can infer that some biochemical activities including Se are perhaps excluded from those associated with S. As observed from the periodic table, the Se atom is larger than S with a radius of 0.5 Å compared to 0.37 Å, for S. Consequently, the bond between two Se atoms is almost one-seventh longer and one-fifth weaker than the disulphide bond *(Sors et al*, 2005b). $SO_4^{2-}$ and $SeO_4^{2-}$ competed for influx to plant roots (Shennan et al., 1990), and exhibited similar Michaelis constants (Km) for high-affinity transport ($K_m$ =15–20 $\mu$M), which was historically observed. However, when plants are supplied with mixtures of $SO_4^{2-}$ and $Se_4^{2-}$, the Se/S concentration ratio in shoot tissues is rarely identical to the Se/S concentration ratio in the rhizosphere (*White et al.*, 2004). In fact, there is often no correlation between the shoot Se and S concentrations of various plant species (or even ecotypes of the same species) growing in the same environment (*Feist* and *Parker*, 2001), although strong relationships between shoot Se and S concentrations have been announced when the analysis was limited to Se non-accumulator crop plants (*Hurd-Karrer*, 1937), as pointed out by *White et al.* (2007).

The Se:S accumulation ratio is obtained by S supply, suggesting that the sulphate transporters induced by S deficiency are more selective for $SO_4^{2-}$ than the sulphate transporters present. Taken together, these observations suggest that several sulphate transporters, with contrasting anionic selectivities, facilitate the uptake of $SO_4^{2-}$ and $SeO_4^{2-}$ by plant roots and that the complementation of these is determined genetically and may be regulated by the plant's nutritional situation. However, the structural basis of the anionic selectivity of different sulphate transporters is unknown. Following uptake by root cells, S and Se are converted into $SO_4^{2-}$ and $SeO_4^{2-}$, which are then loaded into the xylem and transported to the shoot, where they are assimilated into organic composites. Most $SO_4^{2-}$ assimilations take place in the shoot, and the enzymes

responsible are generally encoded by wide gene families, whose products are directed to various intracellular compartments (*Hawkesford*, 2005).

An enhancement in the expression of genes encoding these enzymes is generally observed during S starvation (*White et al.*, 2007). Selenate is accumulated in plant cells against an electrochemical potential (or gradient) by active transport driven by ATP (ATPase). $SeO_4^{2-}$ readily competes with the uptake of $SO_4^{2-}$, and both anions become clear to be taken up by a number of sulphate transporters in the root plasma velum (*Abrams et al.*, 1990). The sulphate transporters modulate Se uptake in bacteria and yeasts, and at least two kinds of these transporters are also present in plants.

The characterized S/Se transporters belong to two major classes (*de Filippis*, 2010): 1) Transporters that have high affinity for sulphate (HAST), which is likely to be the primary transporter involved in the sulphate uptake from the soil, and it is expressed mainly in roots with a Km for sulphate of 7–10 $\mu$M. HAST is also considered to be involved in selenate uptake; and 2) Transporters with a low affinity for sulphate (LAST), which secondary transporter is more likely to be included in the intercellular transport of sulphate, expressed in both the roots and shoots with a Km for sulphate of 100 $\mu$M. LAST is also regarded to be involved in selenate uptake (*Cherest et al.*, 1997). Hence, we may conclude that the physical and chemical similarities of Se and S help to describe the close relationship between Se and S metabolism in plants. Both S and Se are part of Group 16 in the periodic chart; the Se atom is larger than S with a radius of 0.5 Å compared to 0.37 Å for S. Several sulphate transporters, with contrasting anionic selectivities, facilitate the uptake of $SO_4^{2-}$ and $SeO_4^{2-}$ by plant roots, and the complementation of these is genetically determined and may be regulated by the plant's nutritional status.

## 3 Application of selenium in fertilizers

Inorganic Se fertilization on a national scale has proven its efficiency in Finland since 1984, when the interpolation of Se into all multi-element fertilizers became compulsory. Se concentrations in Finnish foodstuffs have since increased dramatically (*Ekholm et al.*, 2007). Soil, climatic, and cropping situations will affect the adequacy of Se biofortification; experiences gained in Finland and other places may not be appropriate to other regions. Another important factor to remark is that the window of Se intake from deficiency to toxicity is rather narrow, necessitating detailed studies on the efficacy of Se biofortification through fertilization if this approach is to be adopted on a commercial

scale (*Broadley et al.*, 2010).

In controlled environmental studies, growth stimulations induced by $SeO_4^{2-}$ fertilization have been reported in ryegrass (*Xue & Hartikainen*, 2000; *Cartes et al.*, 2010), lettuce (*Ríos et al.*, 2009), potato (*Turakainen et al.*, 2004), arabidopsis (*White et al.*, 2004), and soybean (*Djanaguiraman et al.*, 2005). Growth or yield stimulation may be because of selenate-induced antioxidant production such as ascorbate and glutathione (GSH) peroxidases that detoxify $H_2O_2$ and improve stress resistance (*Ríos et al.*, 2009). Selenate-induced upregulation of sulphate transport and merger is also likely to happen (*Van Hoewyk et al.*, 2008). Hence, whilst Se is probably beneficial to vascular plants, as to our knowledge, no growth in yield or stress resistance has been observed in Se-enriched field-grown crops (*Broadleyet al.*, 2010).

Different ways of raising Se concentrations have been investigated over the years to overcome the naturally low Se content of crops in some areas, and this subject has been discussed in a number of reviews (*Gissel-Nielsen & Gupta*, 2004). It is well known that in Finland in 1984 Se-containing fertilizers came into public use. Sodium selenate is added to the fertilizer slurry in order to obtain an equal Se concentration in the granules during the process of production (*Hartikainen*, 2005). Since the beginning of Se fertilization, its impact has been regularly monitored by analysing Se in agricultural soils, water and plants, all kinds of feeds, plant and animal foods, and human serum, the results of these works appearing in numerous publications such as *Ekholm et al.* (1994) or *Eurola et al.* (2003). The Se amount in fertilizers has been regulated on the basis of these findings. The primary level of 16 mg $kg^{-1}$ used for cereal crop fertilizers was decreased to 6 mg $kg^{-1}$ (in 1991). Since this measure had an adverse effect on the crop quality, the Se concentration was increased to the present level of 10 mg $kg^{-1}$ (in 1998).

Fertilization induced severe changes in the Se concentration of agricultural crops. For instance, in spring cereals, the increase was generally 20–30-fold during the first years of supplementation. The Se amount in 2005 was about 13 times higher than in the mid-1970s. In winter cereals, the Se levels increased at first 2–5-fold to 0.07 mg $kg^{-1}$ dry weight in 1990, the present level being about 10–12 times higher than that in the 1970s, as pointed out by *Hartikainen* (2005).

The first studies on the soil application of Se in the 1960s involved the spraying of selenite or selenate solutions onto the soil surface. These relatively simple experiments from New Zealand and USA showed much promise to increase Se content through such treatments, and they have been followed by extensive studies all over the world (*Gissel-Nielsen & Gupta*, 2004). On the

other hand, a large-scale field experiment involving an annual addition of 60 and 120 g Se $ha^{-1}$ over a period of 5 years (as $Na_2SeO_3$) incorporated into a NPK compound fertilizer was carried out in 21 farms covering the common Danish soil types. The soils varied in their content of organic matter, clay, prior cropping, etc. but were all glacial deposit mineral soils with a pH of 5–7. The 120 g Se treatment increased the native Se concentration of 0.02–0.04 mg $kg^{-1}$ of wheat, barley, rye grass, clover, and fodder beets (0.09 mg $kg^{-1}$ in the beet top) to 0.08–0.13 mg Se $kg^{-1}$, which is considered a sufficient and safe level for animal nutrition (*Gissel-Nielsen & Gupta*, 2004). It has been reported that field experiments were carried out on two South Australian sites (*Charlick & Minnipa*, 2002), where Se was applied as sodium selenate at rates from 0 to 120 g $ha^{-1}$ Se either to the soil upon seeding or as a foliar spray after flowering (*Lyons et al.*, 2005).

Applications of $Na_2SeO_3$ to soils or as a foliage spray are proposed for correcting Se nutritional deficiencies in areas with low soil Se. However, in view of the toxic properties of Se salts, these practices should be carefully controlled and the surplus of Se to soil, at 10 g $ha^{-1}$, affected its contents in grains of barley and oat, from 0.019 to 0.26 mg $kg^{-1}$ and from 0.032 to 0.44 mg $kg^{-1}$ respectively (*Gupta & Gupta*, 2000). Soil applications are advised in general, particularly for crops bent due to late-season moisture or heat stress (*Lyons et al.*, 2005), although foliar applications can also be efficient (*Ducsay & Ložek*, 2006) due to the mobility of Se in plants (*Broadley et al.*, 2010).

## 4 Selenium treatment of seeds

Among the three major methods of Se enrichment, seed treatment has been studied the least. Field experiments have shown that seed treatment with Se offers great promise for enriching soybeans (*Glycine max* Merr L.), which are rather high accumulators of Se. Recent data has shown that at equivalent rates of seed-applied Se, soybean grain contained higher Se than a number of other feed and food products (*Gissel-Nielsen & Gupta*, 2004). The effects of different rates of seed-applied Se for two soybean cultivars have been examined. The results demonstrated that increasing Se from 10 to 100 g $ha^{-1}$ proportionately also increased Se concentration in the grain. Thus, grains containing up to 7.5 mg Se $kg^{-1}$ obtained at an application rate of 100 g Se $ha^{-1}$ should not pose a toxicity danger. Due to the higher capacity of soybeans to mobilize Se in the grain, seed treatment with Se offers an alternative for producing crops with the desired Se amounts (*Gissel-Nielsen & Gupta*, 2004). Therefore, considering

all previous experiments, the overall conclusion is that a foliar application of about 5 g Se $ha^{-1}$ as $SeO_3^{2-}$ or $SeO_4^{2-}$, soil fertilization using about 10 g Se $ha^{-1}$ as $SeO_4^{2-}$ or about 120 g Se $ha^{-1}$ as $SeO_3^{2-}$, and 10 g $SeO_4^{2-}$ Se $ha^{-1}$ as seed treatment are efficient annual treatments for increasing the Se content of annual crops to a favourable level for human and animal nutrition. The effect of Se is enhanced when it is used with a detersive for foliar application. The greatest impact can be reached when performed on a well-established crop for all treatments. The remaining effect of these treatments is very small and a higher amount is needed for pasture crops, but this gives a residual effect lasting 2–3 years.

## 5 Selenium in edible parts of plants

Se concentrations in plant foods, such as wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.), may largely vary among countries and regions. Thus, to avoid Se deficiency and toxicity, it is important to monitor and optimize crop Se concentrations (*Zhu et al.*, 2009). In 2009, a universal survey of Se on rice purchased from retail outlets highlighted that Se levels in main rice-producing and -consuming countries, such as Egypt, China, and Thailand, are low, whereas they were higher in rice produced in the USA and India. The concentration of Se in wheat also shows large regional variations (*Hawkesford & Zhao*, 2007). Where both rice and wheat are produced (e.g. India, China and Egypt), the Se concentrations of wheat and rice tend to be similar.

Offsetting regions with inadequate Se by sourcing Se-rich grain is a practical solution to ease the problem, but further characterization of both rice and wheat grain Se concentrations is required (*Williams et al.*, 2009). The Se amount of crops has received much attention recently owing to its importance in the food chain, as mentioned before. Most existing data are related to food and fodder plants. In general, the mean concentrations of Se in grains are higher in countries with dry climates than in countries with humid climates. The average range of mean Se amounts varies from 0.34 to 0.92 mg $kg^{-1}$ for countries with high Se amounts in grains and from 0.014 to 0.042 mg $kg^{-1}$ for countries with low Se amounts in grains. These variations do not suggest a significant effect of climatic conditions because several other factors also control the Se absorption by plants (*Kabata-Pendias*, 2011). It has been found that the environmental trace of the Se concentration in broccoli was about 10 times higher than the genotype impact. A change in the Se uptake by various species of the same plant (Astragalus) is described by Somer and

Caliskan (2007). It was also found that most plants have rather low Se levels, around 25 $\mu$g kg$^{-1}$ and seldom exceed 100 $\mu$g kg$^{-1}$. However, some plants show great capability to accumulate Se, and they may concentrate Se to extremely high amounts, which may be toxic to humans and animals. As mentioned before and according to *Kabata-Pendias*' review in 2011, although Se is not a necessary element for plants, with some exceptions, it is added to soil to ensure that both food and feed crops include enough amounts for dietary needs.

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# The essentiality of selenium for humans, animals, and plants, and the role of selenium in plant metabolism and physiology

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**Abstract.** After its discovery, selenium was most noted for its harmful effects. Selenium was the first element identified to occur in native vegetation at levels toxic to animals. Poisoning of animals can occur through consumption of plants containing toxic levels of selenium. Livestock consuming excessive amounts of selenized forages are afflicted with "alkali disease" and "blind staggers". Typical symptoms of these diseases include loss of hair, deformed hooves, blindness, colic, diarrhoea, lethargy, increased heart and respiration rates, and eventually death. On the other hand, selenium deficiency in animal feeds can cause "white muscle disease", a degenerative disease of the cardiac and skeletal muscles. In this regard, this review paper attempts to summarize the essentiality of selenium for humans, animals, and plants and the role of selenium in plant metabolism and physiology.

**Keywords and phrases:** selenium deficiency, essentiality of selenium, selenium in plant metabolism and physiology

# 1 Essentiality of selenium for humans, animals, and plants

Perceptions of selenium changed when *Schwarz & Foltz* in 1957 reported that additions of selenium prevented liver necrosis in rats (*Rattuss* pp.) deficient in vitamin E. Its role in human health was established in 1973, when selenium, the last of 40 nutrients proven to be essential, was shown to be a component of glutathione peroxidase (GSHx), an enzyme that protects against oxidative cell damage. The United States' recommended daily allowance for selenium is 50 to 70 $\mu$g in human diets. Currently, all of the known functions of selenium as an essential nutrient in humans and other animals have been associated with selenoproteins (*Kopsell & Kopsell*, 2007).

The essentiality of Se for higher plants is still under debate, but Se is considered a beneficial nutrient for many plant species (*Pilon-Smits et al.*, 2009) – maybe for better oxidative stress resistance (*Hartikainen*, 2005). Plants readily take up and assimilate Se, a capacity that may be used to diminish both Se deficiency and toxicity in animals and humans. Plants can be used to clean up surplus Se from polluted areas (phytoremediation), and Se-enriched plant material may be considered fortified food (biofortification) (*El Mehdawi et al.*, 2012; *El-Ramady et al.*, 2014b). It is well known that the element Se is considered a limited and not renewable resource on earth. It is a necessary element in humans, animals, microorganisms, and some other eukaryotes; but as yet its necessity to plants is in dispute. However, Se has not been approved to be an essential microelement to artery plants. There are some documents that Se may be essential for growth and development in algae (*Pilon-Smits et al.*, 2009). Also without any doubt, adequate amounts of Se are significant to animal and human health, and some Se compounds have been found to be active against cancers. A limited number of plants growing on Se-enriched soils can accumulate very high amounts of Se (i.e. hyperaccumulate Se) and are classified as Se tolerant; however, many more plants do not accumulate Se to any excess extent and are Se sensitive. Plants vary considerably in their physiological and biochemical reply to Se, and a revision of the physiological reply of plants to Se is presented; especially growth, uptake, transport, and interplay of Se with other minerals, as pointed out by *de Filippis* (2010).

Even in the best-studied Se-accumulating plant, *Astragalus pectinatus*, the results of additional Se application in experiments have had differing results (*Stadtman*, 1990). It is fair to point out that other nutrients can complex the situation, such as sulphates, phosphates; however, the experiments so far have not used controls where residual Se is not present at all; and indeed such

experiments may be nearly impossible to perform (*Stadtman*, 1996).

This is simply because there will always be trace amounts of Se in plants, coming from impurities in the nutrients used or even coming from the atmosphere. A substitute approach trying to resolve essentiality was an attempt at specifying Se interpolation into Se-dependent enzymes, with an integral Se-Cys residue as present in animals and bacteria (*Axley et al.*, 1991). As *Filippis* (2010) contends, the available evidence from molecular studies so far is quite strong that there is no clear evidence for necessary selenoproteins in higher plants, but part of the machinery for the synthesis of selenoproteins may be present in plants.

It is well known that Se is a contradictory nutrient since it is called *the essential poison* – too much of it in the diet can be toxic, while too little can result in chronic and sometimes fatal deficiency (*Reilly*, 2006). Organisms that require Se for normal cellular function contain necessary selenoproteins, such as glutathione peroxidase, formate dehydrogenase, and selenophosphate synthase. Interestingly, the interpolation of selenocysteine into these selenoproteins is directed by a specific tRNA that recognizes a UGA-opal codon (*Ellis* and *Salt*, 2003). The UGA codon normally acts to terminate translation. In combination with a selenocysteine insertion sequence (SECIS), the UGA codon is identified by the selenocysteine tRNA, which manages the insertion of selenocysteine (*Low* and *Berry*, 1996). There is not any strict evidence for the specific incorporation of selenocysteine in vascular plants. Various selenoproteins that involve a glutathione peroxidase homologue and selenocysteine tRNA have been specified in the plant system of Chlamydomonas reinhardtii (*Fu et al.*, 2002). And as suggested by *Ellis & Salt* (2003), evidence for the specific insertion of selenocysteine in vascular plants is less certain. Therefore, it could be concluded that the necessity of Se for higher plants is still uncertain, but Se is marked as a beneficial nutrient for many plant species.

## 1.1 Is selenium physiologically important for higher plants?

Se has not been known as an essential element for higher plants as yet, although its role has been regarded to be beneficial for plants that are capable of a large-scale accumulation of this element (*Shanker*, 2006). According to *Hamilton* (2004), the role of Se in plants mainly depends on its concentration. Se has three levels of biological activity: trace concentrations are essential for normal growth and development, moderate concentrations can be stored to maintain homeostatic functions, and elevated concentrations can eventuate in toxic effects, as *Fig. 1* shows.

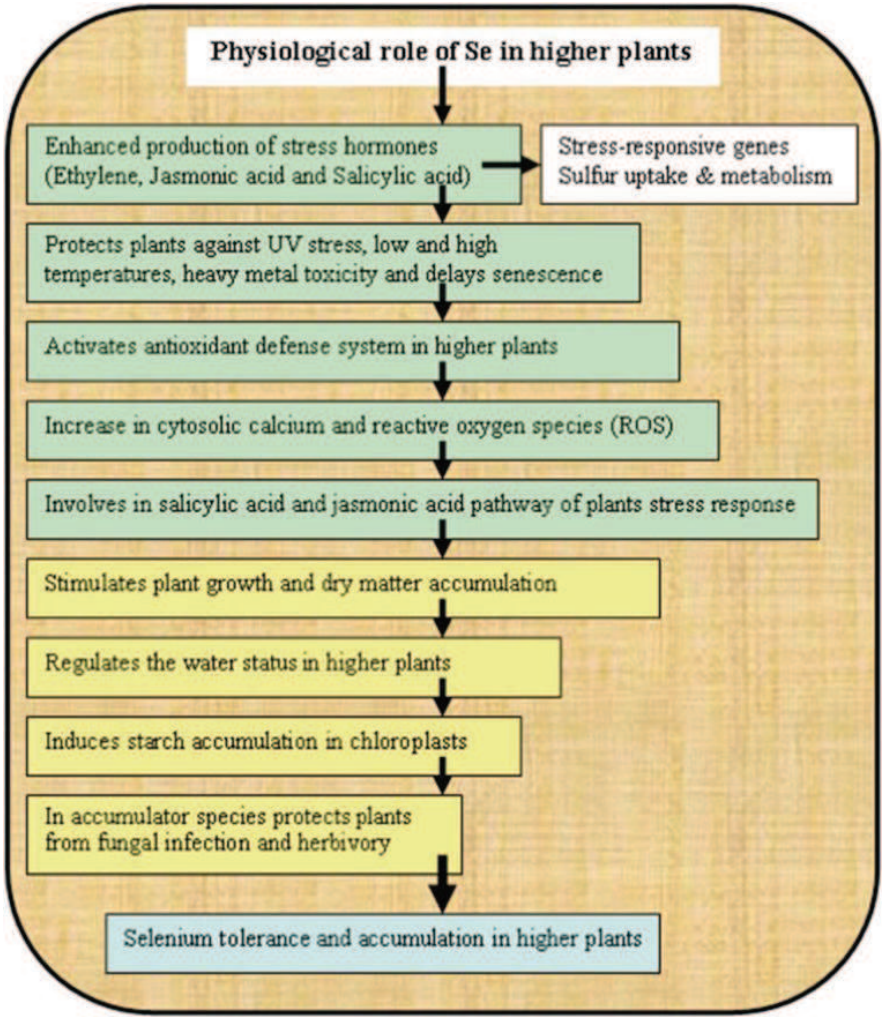


Figure 1: Physiological functions or roles of Se in higher plants (*Hasanuzzaman et al.*, 2010; *Hajiboland*, 2012; *El-Ramady et al.*, 2014)

*Combs & Combs* (1986), *Germ & Stibilj* (2007), and *Pilon-Smits* (2015) investigated in many studies the function of Se in plants, and they found that there is little evidence for the essentiality of Se for all plants. *Hartikainen* (2005) did several studies on some grasses and vegetables and indicated that with a proper Se addition the growth rate of these plants may be increased. Certain data indicated that this element may be required for

Se-accumulating plants (*Moxon* and *Olson*, 1974). Some Se compounds with cysteine and methionine were found in such plants such as *Astragalus* species, but their metabolic functions have not been definitely established. Whereas Se-accumulator plants synthesise Se-methyl-cysteine, non-accumulator species produce Se-methyl-methionine. *Kabata-Pendias* (2011) pointed out that the physiological significance of this difference has not been not identified yet.

Several selenoamino acids, selenomethionine (SeMet), selenocysteine (Se-Cys), and Se-methylselenocysteine (SeMSC) in association with glutathione peroxidases were found in both bacteria and higher plants (*Kabata-Pendias*, 2011). Selenocysteine, methylselenocysteine, selenomethionine, selenotaurine, selenobetaine, selenoecholine, dimethylselenine, dimethyldiselenide, and trimethylselenium are different Se species in plants (*Pyrzynska*, 1995). For example, SeMet is a predominant form of Se in cereal grains, legume seeds, and lentils (up to 95% of total Se), while SeMSC in vegetables (*Djujic et al.*, 2001).

*Singh et al.* (1980) were the first ones to write about the positive effect of Se on plant growth. They showed the application of 0.5 mg $kg^{-1}$ Se, as selenite stimulated the growth and dry matter yield of Indian mustard (*Brassica juncea* L.). They also found that Se applied in low concentrations can increase the growth and antioxidative volume of both mono- and dicotyledonous plants. *Hartikainen et al.* (1997) demonstrated a positive response of lettuce (*Lactucasativa* L.) growth to Se, while *Djanaguiraman et al.* (2005) obtained the same results in relation to soybean (*Glycine max* L.).

At a higher supplementation level than 29 mg $kg^{-1}$ soil, Se inhibited the growth and germination of tomato, lettuce, and radish (*Raphanus sativus* L.) seeds (*Carvalho et al.*, 2003). Hence, Se has an effect on germination. But according to *Hasanuzzaman et al.* (2010) the positive effect on germination was linked to antioxidative activity, and selenite improved germination of bitter gourd (*Momordica charantia* L.) seeds at sub-optimal temperatures (*Chen* and *Sung*, 2001).

Se could be used for the phytoremediation in Se-contaminated fields. Plants that have a high capacity to accumulate and tolerate Se are suitable for it (*Terry et al.*, 2000). But, generally, most plants have a low tolerance to high Se amounts, and they contain less than 25 $\mu$g Se $g^{-1}$ dry weight (DW), being considered as non-accumulators (*White et al.*, 2004). Non-accumulators are susceptible to high Se concentration, but tolerance and accumulation of Se, even at high concentrations, is possible for them without reduction in growth (*Rani et al.*, 2005).

The critical Se concentration in plant tissues, which decreased the yield in Indian mustard, was 105 $\mu$g $g^{-1}$ DW, in maize (*Zea mays* L.) 77 $\mu$g $g^{-1}$ DW,

in rice (*Oryza sativa* L.) 42 $\mu$g g$^{-1}$ DW, and in wheat 19 $\mu$g g$^{-1}$ DW, when Se additions, such as selenite, were 5, 5, 4, and 10 $\mu$g g$^{-1}$ soil for Indian mustard, maize, wheat, and rice respectively (*Rani et al.*, 2005). Depending on the plant species, growth stage, and plant organs, Se uptake and metabolism will be different. Broccoli (*Brassica oleracea* var. *italica*) is known for its ability to accumulate high levels of Se, with a greater number of the selenoamino acids in the form of Se-Met (SeMeSeCys) (*Lyi et al.*, 2005). Most plants accumulate more Se in shoot and leaf than in root tissues, but there are exceptions (*Zayed et al.*, 1998). Se concentrations in the higher leaves, roots, stolons, and tubers of potato increased with increasing Se supplementation (*Turakainen*, 2007). The highest Se concentration was observed in young upper leaves, roots, and stolons, and indicated that added selenate was efficiently utilized and taken up at an early stage. The Se concentration declined in the upper parts, roots, and stolons of potato plants during the growing period, whereas an intensive accumulation happened in immature and mature tubers (*Turakainen*, 2007).

Se accumulation was also affected by the methods of application, where foliar application with selenate significantly increased Se content in tea leaves (*Hu et al.*, 2003). According to other results, the Se content of pea seeds obtained from untreated and once or twice foliar-treated plants was directly proportionate to the number of sprayings (*Smrkolj et al.*, 2006). Several studies proved that Se is taken up from the soil by plants primarily as selenate ($SeO_4^{2-}$) or selenite ($SeO_3^{2-}$) (*Ellis* and *Salt*, 2003). Due to the faster incorporation of selenite than selenate, a higher toxicity of selenite compared to selenate has been suggested (*Lyons et al.*, 2005). The uptake of selenate into roots and its dispersion in plants is much faster than that of selenite (*Cartes et al.*, 2005). The total Se accumulation in a plant was about tenfold higher from selenate compared to selenite, as *De Souza et al.* (1998) reported.

What we can conclude from all outcomes is as follows: Se as an essential element for higher plants has not yet been classified, but it has an important beneficial role for plants that can accumulate large amounts of this element. Although the essentiality of selenoproteins in higher plants has not been proved, syntheses of selenoproteins in some plants, e.g. sugar beet, were reported. Se, at low concentrations, increases the growth and antioxidative capacity of both mono- and dicotyledonous plants. The physiological importance of Se for higher plants could be evaluated within the following topics: anti-oxidative and pro-oxidative effects of Se and the role of Se under abiotic stresses.

### 1.1.1 The role of selenium in plant metabolism and physiology

Environmental selenite, which is prevalent in reducing environments, and selenite, which is prevalent in toxic environments, are taken up non-specifically by plants typically using transporters for sulphur (S) analogues. Via the sulphate assimilation pathway into selenocysteine (SeCys), selenomethionine (SeMet), and other organic S compounds, these inorganic forms of Se may be assimilated. This process can happen in the shoot, but it is thought to take place in the root. When seleno-amino acids get incorporated into proteins, replacing Cys and Met, this accidentally impairs protein function and thus results in toxicity (*Stadtman*, 1990). Most plants can metabolize SeMet into volatile dimethylselenide (DMSe), which may help avoid toxicity (*Terry et al.*, 2000). Another potential Se detoxification mechanism in plants is the breakdown of SeCys into elemental Se and alanine (*Van Hoewyk et al.*, 2005; *Prokisch et al.*, 2008). Both volatilization and separation of SeCys are non-specific, using enzymes that function in S metabolism. *Fig. 2* summarizes Se metabolism in plants (*Terry et al.*, 2000; *Van Hoewyk et al.*, 2007; *Pilon-Smits*, 2015).

Not only these general mechanisms take place in plants, which metabolize Se inadvertently, but some plants may also be able to discriminate between Se and S analogues, and so these can be said to have Se-specific metabolism. For instance, these plants can methylate SeCys into methyl-SeCys, which serves as a very effective Se detoxification mechanism since methyl-SeCys does not get combined into proteins (*Neuhierl* & *Böck*, 1996). This methylation process is mediated by the enzyme SeCys methyltransferase (SMT). Plants that contain this enzyme are called Se hyperaccumulator and can fill up to 1.5% of their dry weight as Se (15,000 mg $kg^{-1}$ DW, *Beath et al.*, 1939a, b). However, SMT was also detected in broccoli (*Brassica oleracea*) (*Lyi et al.*, 2005), and methyl-SeCys was found in several *Allium* species such as garlic (*Ge et al.*, 1996). Although these species are known to be sulphur-loving, they are not hyperaccumulators, but due to high levels of sulphate that they accumulate the amount of their Se uptake is remarkable. Hence, sometimes they are known as Se-accumulator plants.

Hyperaccumulators of Se have several properties that separate them from other species. True Se hyperaccumulation occurs in 4–5 genera in the Brassicaceae, Fabaceae, and Asteraceae. They occur predominantly or even exclusively on seleniferous soils (*Beath et al.* 1939a, b). They accumulate ∼100-fold higher Se levels and have higher tissue Se/S levels than the surrounding vegetation (*Lauchli*, 1993). Hyperaccumulators accumulate organic forms such as

methyl-SeCys and selenocystathionine, whereas most plants accumulate inorganic Se (*Anderson*, 1993).

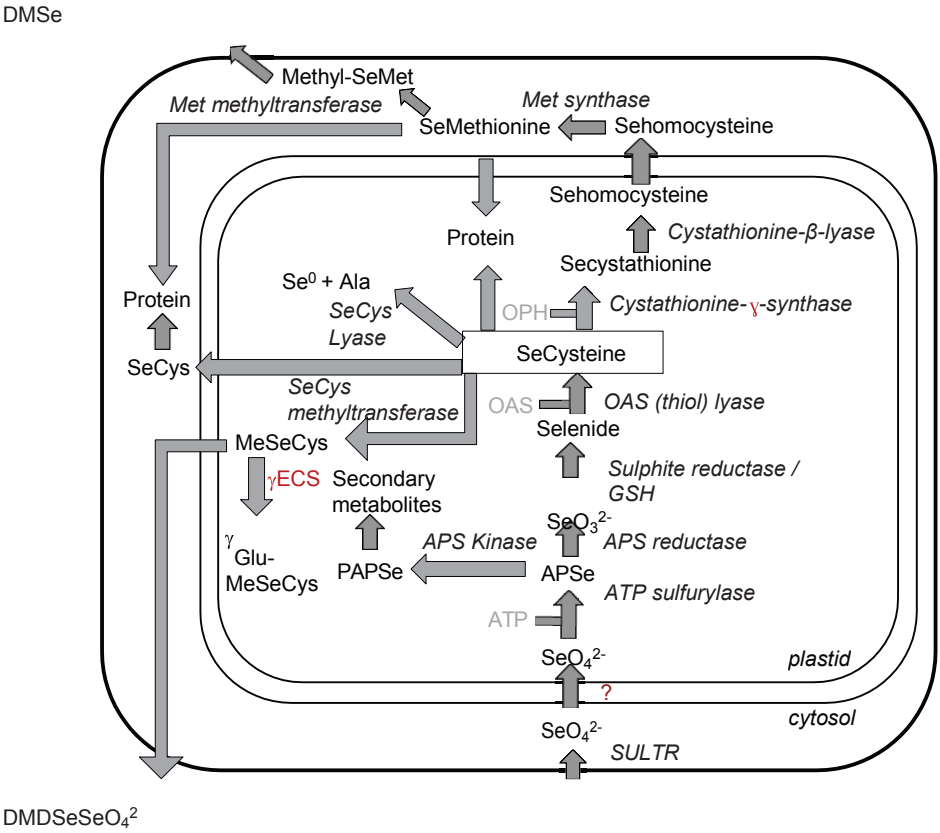


Figure 2: Proposed model for Se assimilation in plants

Enzymes are shown in italic and metabolites in black or grey. SULTR – sulphate/selenate transporter, APSe adenosine phosphoselenate, PAPSe phospho adenosine phosphoselenate, OAS O-acetylserine, OPH O-phospho-homoserine, SeCys selenocysteine, (Se)Met (seleno) methionine, Ala alanine, MeSeCysmethyl-SeCys, gGlu-MeSeCysg-glutamyl MeSeCys, gECSg-glutamylcysteine synthetase, GSH glutathione, DMSe dimethyl-selenide, DMDSe dimethyldiselenide

Hyperaccumulators are completely tolerant to their extreme Se levels and often even grow better under high Se conditions than without Se because organic forms of Se do not interfere with S metabolism (*Broyer et al.*, 1972; *El*

*Mehdawi et al.*, 2012). Hyperaccumulators can volatilize Se, but mostly in the form of dimethyldiselenide (DMDSe), which originates from methyl-SeCys, as in the case of other plants (*Terry et al.*, 2000). Selenium hyperaccumulators also show tissue-specific and organ-specific sequestration patterns that are different from other plants.

Depending on non-accumulators, a larger fraction of the Se in hyperaccumulators is relocated from the root to shoot; also, a larger fraction is remobilized from older leaves to young leaves and reproductive organs, particularly pollen and ovules (*Quinn et al.*, 2011a, b). Through leaves, hyperaccumulators store most of their Se in the vacuoles of epidermal cells, which may include the trichomes (leaf hairs) (*Freeman et al.*, 2006, 2010). For comparison, non-hyperaccumulator *Arabidopsis thaliana* and *Brassica juncea* were found to store most of their Se in the form of selenate in the vascular bundles and to contain higher Se levels in leaves than in floral tissues (*van Hoewyk et al.*, 2005; *Freeman et al.*, 2006; *Quinn et al.*, 2011a). Interestingly, selenate uptake in Se hyperaccumulators is not inhibited by sulphate, suggesting that they have a selenate-specific transporter; this is in sharp contrast to the non-hyperaccumulator *B. juncea* and may explain the elevated Se/S ratios that are typical for hyperaccumulators (*White et al.*, 2007). In like manner, Se and S remobilization in hyperaccumulators follows diverse patterns both developmentally and seasonally (*Galeas et al.*, 2007; *Quinn et al.*, 2011a). Selenium levels are highest in young leaves and reproductive tissues, while S levels are highest in mature leaves. Leaf Se levels in the field peak in early spring, while leaf S levels sharpen in midsummer. In non-hyperaccumulators, both Se and S amounts peaked in midsummer (*Galeas et al.*, 2007; *Pilon-Smits*, 2015).

### 1.1.2 Accumulator to non-accumulator plants

*Rosenfeld* and *Beath* (1964) and *Shrift* (1973) divided plants into three groups on the basis of their ability to accumulate Se when grown on high Se soils. The first two groups of plants are referred to as *Se hyperaccumulatoror indicator plants*. These grow well on soil containing high levels of available Se, and some have been used to locate seleniferous soils. Plants in Group 1 are called *primary indicators* and include many species of *Astragalus*, *Machaeranthera*, *Haplopappus*, and *Stanleya*. These species absorb high concentrations of Se, which might mean hundreds or, occasionally, even thousands of milligrams dry weight per kilogram. Plants in Group 2 are referred to as *secondary Se absorbers*. They belong to a number of genera including *Aster*, certain species of *Astragalus*, *Atriplex*, *Castilleja*, *Grindelia*, and *Gutierrezia* and certain species

of *Machaeranthera* and *Mentzelia*. They rarely concentrate more than 50 to 100 mg Se $kg^{-1}$. Plants in Group 3 include grains, grasses, and many forbs that do not usually accumulate Se in excess of 50 mg Se $kg^{-1}$ when grown on seleniferous soil.

Some plants growing on seleniferous soils accumulate surprisingly low levels of Se. White clover (*Trifolium repens* L.), buffalo grass (*Buchloe dactyloides* [Nutt.] Engelm.), and grama (*Bouteloua* sp.) are poor accumulators of Se. On the other hand, high sulphur-containing plants, such as the *Brassica* sp. (mustard, cabbage, broccoli, and cauliflower) and other *Cruciferae*, are relatively good concentrators of Se (*NAS-NRC*, 1983). Absorption of Se and S by plants may be correlated with each other (*Shrift*, 1973).

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# Selenium in soil–plant–food systems

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**Abstract.** Humans and animals require a multitude of nutrients in order to have a properly functioning body for purposes of growth, development and metabolism. Plant-based foods have represented one of the most important nutrient sources in human diet since the beginning of mankind. But nowadays the amount of arable land is being reduced and much of the natural resources already in use show signs of degradation. Also, staple crops (i.e. plants that constitute the main food in the diets of people in developing countries, e.g. wheat, rice, maize, and cassava) regrettably contain low amounts of micronutrients, making them insufficient to meet the minimum daily requirements. Shortages in mineral micronutrients, including iron (Fe), zinc (Zn), selenium (Se), and iodine (I), are affecting more than half of the world's population. In this case, it is fundamental to improve strategies that let us make plant foods more efficient and with higher micronutrient amounts and bioavailability concerning their edible textures. In this regard, in this review paper, we tried to summarize selenium availability and its application in the soil, plant and food systems to understand the place of selenium in plant-based foods.

**Keywords and phrases:** selenium, soil–plant–food systems, mineral micronutrients, selenium cycling

# 1 History of selenium research

The first interest in Se related to its toxicity and the early work on Se was summarized by *Moxon & Rhian* in 1943, and Se research began in 1817. That year, Berzelius discovered this element and published his first detailed description about the research in 1818. He named the element Selenium. The history of Se research could be considered an attempt and it is made to take a "bird's-eye" view at the development of this research field from 1817 till today (*Arnér*, 2012). The tool chosen is an analysis of the scientific literature on Se research, thereby attempting to give an unbiased assessment of this research field. Lastly, as in all investigations of historic trends, we should also ask where the future of Se research might take us. By necessity, the answer to that question is uncertain. However, it could be concluded that never before has Se research been as intense and expanding as it is today, which also holds major promises for the future (*Arnér*, 2012). Although as early as from 1842 a document was retrieved on the toxicity of Se, the first authentic written record of Se toxicity in livestock was apparently the report by Madison in 1856, who was a military surgeon stationed at Fort Randall, which was then in the Nebraska territory. He described a deadly disease among horses grazing in certain areas near the castle (*Whanger*, 2002). Many reviews have explained the development of Se research, and several findings have shaped today's knowledge in the field, containing personal recollections by some of the pioneers of Se research. I could simply repeat information that have been given in previous reviews on the Se research field.

Hence, the reader is referred to other papers on the history of selenium research for debates on specific details or topics of that research (*Arnér*, 2012).

The first year covered by the ISI Web of Science database (1945) includes eight articles using the keyword selenium. These articles covered the topics of Se poisoning (three articles), Se amounts in soil, plants, or animals (two articles), or the photodynamic exclusivities of Se, its spectral virtues, or the oxidizing valence of dioxide of selenium (one article each). The subjects of those eight papers from 1945 that centred on the agricultural, physical, or chemical properties of Se are for the most part the very same subjects that have made "selenium" a much hotter topic in research than the issue of the more special "selenocysteine" or "selenoprotein" (*Arnér*, 2012).

With the industrial application of Se in ceramics, solar cells, glass, photocopiers, rectifiers, and many more and because of its properties as a catalyst in non-organic chemistry, a great number of research publications on Se are not related to biology or biochemistry at all (*Arnér*, 2012).

It somehow stands to reason to look back and discuss how the history of Se research has evolved. What results will the same analysis give if performed 10 years from now or in 50 or 200 years? Indeed, we cannot know how the future of selenium research will turn out, but we may be sure it will be an exciting one. With this topic of research presently being under quick development, it is clear that the potential of new Se-related discoveries of major importance awaits around the corner, as suggested by *Arnér* (2012).

## 2 The chemistry of selenium

Selenium is one of the rarest elements on the surface of earth (0.05 mg $kg^{-1}$), whereas it is the $69^{th}$ among the 88 elements (*Shriver* and *Atkins*, 1999). Se is named the two-faced element (like the moon, where its name originates from) as it has both a dark and a bright side. It is also known as the "necessary poison or the double-edged blade element" based on its poisonous and beneficial effect on human health (*El-Ramady et al.*, 2014). As it is reported, this duality of Se comes from the way to reconcile its apparently inconsistent properties and roles. Nevertheless, these gaps in our knowledge of Se are rapidly being filled by great efforts of an extraordinary array of researchers, working in a range of disciplines, helped by powerful new research techniques and tools (*Reilly*, 2006). It is clear that Se has a 78.96 atomic weight and 34 as atomic number. It is chemically related to other members of the chalcogen group (Group 16/VIA), which includes oxygen (O), sulphur (S), tellurium (Te), and polonium (Po). Therefore, it is classified as a metal-like element, a "metalloid", but elemental Se has several different allotropes (*Chapman et al.*, 2010). This puts Se in an important group of metalloids, elements that are neither completely metals nor non-metals but have chemical and physical properties of both (*Reilly*, 2006). This position accounts for many of its biological interactions with some main elements containing sulphur as well as with arsenic and its neighbour, phosphorus (*Frost*, 1972). As for the external electronic configuration of this element, it is $3d^{10}\ 4s^2\ 4p^4$, with three fully filled inside shells.

Selenium has four capacity states: $-2$ state, which predominates in organic Se composite besides 2, 4, and 6 states. There are nearly 50 Se minerals. The most important and relatively usual ones include: clausthalite (PbSe), klockmanite (CuSe), tiemannite (HgSe), berzelianite ($Cu_2Se$), crookesite (Cu, Tl, Ag)$_2$Se, and ferroselenite ($FeSe_2$) (*Kabata-Pendias*, 2011). Therefore, the union of this element with host minerals, such as chalcopyrite, pyrite, and

sphalerite, is relatively common. On the other hand, this element has a major dependency on different substances resulted in a large number of organic compounds that are analogous to those of S-organic compounds and are quickly accumulated in some biolithes (*Kabata-Pendias*, 2011). As mentioned before, the average content of Se in the Earth's crust is estimated at 0.05 mg $kg^{-1}$; however, a higher amount (up to 0.5 mg $kg^{-1}$) is also given. This element is slightly more concentrated in mafic rocks (rarely trespasses 0.1 mg $kg^{-1}$), while Se is related to clay fractions, and thus, its abundance in argillaceous sediments ranged from 0.3 to 0.6 mg $kg^{-1}$ in sedimentary rocks. This concentration is higher than in sandstones and limestones (0.01–0.1 mg $kg^{-1}$).

On the other hand, due to the fact that Se contains some amino acids (SeMet and SeCys), it is a completely special trace element and therefore involved in very special biological roles. These main roles include conservation against oxidative injuries, defences against infection, and modulation of growth and improvement. Hence, the main exposure to Se occurs through the food chain, and its distribution in natural environments has a specific effect on its content in soils and crops and on human health (*Marmiroli* and *Maestri*, 2008). In agriculture, Se is used especially as sodium selenite ($Na_2SeO_3$) as a supplier to fertilizers, insecticides, and foliar sprays. In small doses, Se is widely used in vitamins, other dietary supplements, and some ruminant feeds (as a fortifying element). Moreover, it is a relatively usual component of different cosmetics and medications as a remedial agent (e.g. in cardiology as an antioxidant) (*Kabata-Pendias*, 2011).

## 2.1 Selenium on earth and selenium cycling

Se is rarely recovered in a free state and exists in different oxidation states with 6 (VI), 4 (IV), 0 (Se0), and -2. The oxidized water-soluble forms that have selenate Se(VI) and selenite Se(IV) can be found in both natural water and soil solution (*Kabata-Pendias*, 2011). High stable elemental selenium ($Se^0$) can again be found in soils, but in aqueous solutions it is not same because it is insoluble (*El-Ramady et al.*, 2014). This elemental selenium ($Se^0$) can exist in distinct allotropic forms, including rhombohedra Se (containing $Se_6$ molecules), three deep-red monoclinic shapes, $\alpha$-, $\beta$-, and $\gamma$-Se (containing $Se_8$ molecules), trigonal grey Se (containing Sen helical chain polymers), black, vitreous Se, and amorphous red Se. The grey (trigonal) Se is thermodynamically the most stable form, which has countless helical chains of Se atoms, and it is the only allotropic form that conducts electricity (*El-Ramady et al.*, 2014). The most presumably amorphous allotrope forms that occur in soils involve

both red and black. Furthermore, red amorphous Se can gradually return to the black amorphous form at temperatures above 30 °C. This black form can then slowly transform into the much more stable grey hexagonal allotrope or, depending on the pH and redox conditions of the soil, it will be re-oxidized (*Di Gregorio*, 2008).

In various environments and due to different processes, selenium spreads e.g. through volcanic activities and hot springs, soil and rock weathering, inflammation of fossil fuels, soil leaching, sea salt spray, forest wildfires, groundwater motions, chemical and biological redox reactions, and mineral shaping, burning of municipal waste, Zn and Pb smelting, Cu/Ni, Fe and steel production, crop growth and irrigation practices, and plant and animal uptake and release (*Di Gregorio*, 2008). As a general law, Se concentration in soils or ground and fresh waters depends upon the source material, mapping, age of the soil, climate, and agricultural or industrial usage. Elemental Se and selenides are two prevailing species under acidic, reducing conditions in soils that are likely waterlogged and rich in organic matter (*Di Gregorio*, 2008; *El-Ramady et al.*, 2014).

## 2.2 Production, sources, and uses of selenium

At the global level, there are no mines that particularly extract Se; in contrast, it is a by-product of the production of other metals such as the refining of Pb and Cu or is recovered from the sludge accumulated in $H_2SO_4$ factories (*Johnson et al.*, 2010). The supply of Se is affected by the supply of the materials whose direct by-product it is – Cu, and, to a lesser extent, Ni. The estimated domestic Se production was slightly higher in 2012 in comparison with 2011 owing to a slight increase in Cu production (1,980 and 2,000 metric tons in 2011 and 2012 respectively; USGS, 2013). On a global level, Japan, Canada, and the USA produce great amounts of selenium, while smaller amounts are coming from Australia, Finland, Peru, Zambia, Belgium, Russia, China, and countries that have industry of Cu refining. Lots of Se compounds, such as cadmium sulfoselenide, ferro- and nickel selenide, selenium dioxide, and selenium diethyldithiocarbamate, are available commercially, as well as sodium selenate and sodium selenite (*El-Ramady et al.*, 2014).

Globally speaking, Se is continually recycled in the environment via the marine, atmospheric, and terrestrial systems. Estimates of Se flux specified that anthropogenic activity (76,000–88,000 tons per year) is a main source of Se release in the cycle, while the marine system (38,250 tons per year) offers the main natural pathway. Because of the rapidity of transport, Se

cycling via the atmosphere (15,300 tons per year) is considerable, but the terrestrial system (15,380 tons per year) is the most important in the case of human and animal health because of the direct relationship with the food chain and the agricultural processes. Although Se is a derivative of both natural and man-made sources, understanding the relationship between environmental geochemistry and health is of particular importance as rocks are the basic source of this element in agroecosystems (*El-Ramady et al.*, 2014).

There are several important agricultural and horticultural applications for Se. These applications involve the use of sodium selenite and selenate as additives and dietary complements in animal forages. Soil deficiencies could be supplemented by addition of Se compounds to fertilizers and top-dressings. On the other hand, in the 1930s, potassium ammonium sulfoselenide, which was used as a pesticidal substrate and was one of the first systemic insecticides to be regarded, became the centre of attention. This compound is still in use, but it is limited to non-food crops because of its poisonous character. In commercial greenhouses growing flowers for cutting, sodium selenate ($Na_2SeO_4$) was also used for similar purposes. This selenate ($Na_2SeO_4$) could be added to irrigation water, and the plant roots can take it up. Then, it is transformed in the leaves into volatile selenide ($Se^{2-}$), which is produced by the plant against aphids, to repel red spiders and similar pests (*El-Ramady et al.*, 2014).

## 3 Selenium in the soil–plant–food systems

It is clear that Se content in soils is inherited from source material and its distribution completely reflects soil-shaping processes and atmospheric deposition. Sandy soils, which developed under humid climate, especially in podzols, have the lowest amounts of Se, whereas the highest amounts are often to be found in organic and calcareous soils (*Kabata-Pendias*, 2011; *Ramady et al.*, 2014). Generally, the concentrations of Se in soils range from 0.05 to 1.5 mg $kg^{-1}$ worldwide, with a calculated average value of 0.44 mg $kg^{-1}$. However, we can also observe higher amounts of Se in the surface layer of volcanic soils, forest soils, organic rich soils, and calcareous soils. In general, the main factors controlling Se forms and behaviour in soils are Eh, or redox potential, and pH; however, a number of other parameters, such as organic ligands, clay content, and hydroxides, also play very significant roles (*Kabata-Pendias*, 2011; *Ramady et al.*, 2014). It has been reported about different inorganic species of Se, which were associated with defined soil parameters, that they reveal variable properties as follows: (1) selenates (mobile in inorganic forms

in neutral and alkaline soils but not absorbed on hydrous sesquioxides, especially $Fe_2O_3 \cdot H_2O$), (2) selenites (slightly mobile in neutral and acid soils of humid temperate regions and are quickly absorbed on hydrous sesquioxides and organic matter), and (3) selenides (rather immobile in acid soils because of the formation of stable mineral and organic compounds) (*Combs* and *Combs*, 1986; *Kabata-Pendias*, 2011; *Ramady et al.*, 2014).

The most significant forms and concentrations of Se in soil solution are governed by different physical, chemical, and biological factors, and usual inorganic anions include $HSeO_3^-$, $SeO_3^{2-}$, $H_2SeO_4^-$, $SeO_4^{2-}$, and $HSeO_4^-$ (*Kabata-Pendias* and *Sadurski*, 2004). Selenate anions ($SeO_4^{2-}$) are the favoured form under oxidizing conditions, whereas under mild reducing conditions $SeO_3^{2-}$ is likely to dominate (*Kabata-Pendias*, 2011). $SeO_3^{2-}$ is strongly sorbed on oxides and precipitates such as $Fe(SeO_3)_3$, whereas $SeO_4^{2-}$ anion is very weakly sorbed, especially at high pH. Therefore, mobile and easily phyto-available (available to plants) Se occurs in well-aerated alkaline soils, which are common in arid and semi-arid regions. On the other hand, organic matter has a strong tendency to shape organometallic complexes to remove Se from the soil solution (*Kabata-Pendias* & *Mukherjee*, 2007). Also the phyto-availability of different Se species in soils declines in the following order: selenate > selenomethionine > selenocysteine > selenite > elemental selenium > selenide (*Kabata-Pendias* & *Mukherjee*, 2007). Moreover, a close relationship is reported between Se and organic carbon in most soils.

Microbial processes play a decisive role in Se cycling in both the formation and mineralization of organic Se such as selenomethionine and selenocysteine and especially in its volatilization from soils contaminated with Se (*Martens* & *Suarez*, 1998; *Ramady et al.*, 2014). These processes are important for the reduction of Se, fundamentally through the reduction of selenate and selenite. Insoluble selenide composites can accumulate in cases of improperly drained soils. Se may volatilize in the form of $(CH_3)_2Se$, just as in the form of several other methane and sulphide Se compounds, due to methylation processes under anaerobic conditions (*Kabata-Pendias* & *Mukherjee*, 2007). A number of microorganisms, such as bacteria and fungi species, are included in the volatilization processes of Se. It is reported that organic corrections may considerably increase the rate of Se volatilization from soils (*Kabata-Pendias* & *Mukherjee*, 2007; *Ramady et al.*, 2014).

Generally, Se content can be increased in plants in various ways, including foliar applications, hydroponics, or aeroponic cultivation in a nutrient solution containing Se and wetting seeds in Se solution before sowing (*Germ et al.*, 2007; *El-Ramady et al.*, 2014). Therefore, the Se uptake by plants (mainly as

$SeO_4^{2-}$ or $SeO_3^{2-}$, when it is present in soluble forms) depends on a number of factors related to soil and plant characteristics, although differences between these plant species are very accentuated (*Kabata-Pendias*, 2011). *Rayman et al.* (2008) reported that there was no bioavailability data for either Se-methyl-selenocysteine or $\gamma$-glutamyl-Se-methyl-selenocysteine.

Although Se has not yet been classified as a necessary element for higher plants, its role has been remarked to be beneficial for plants that are able of uptake followed by large-scale accumulation (*Shanker*, 2006). There are various organic Se species, including selenocysteine, methylselenocysteine, selenomethionine, selenotaurine, seleniobetaine, seleniocholine, dimethylselenide, dimethyldiselenide, and trimethylselenonium (*Pyrzynska*, 1995). The necessity of these seleno-proteins in higher plants has not been proved, but syntheses of them in some plants, such as sugar beet, have been reported (*Terry et al.*, 2000). Furthermore, various seleno-amino acids containing selenomethionine (SeMet), selenocysteine (SeCys), and selenomethylocysteine (SeMC) in relationship with glutathione peroxidases have been found in both higher plants and bacteria (*Kabata-Pendias*, 2011; *Ramady et al.*, 2014).

The uptake and metabolism of Se is different because of the difference in growth stage, plant species, and plant organs. More Se accumulates in shoot and leaf than in root tissues in different plants, but there are some exceptions (*Zayed et al.*, 1998). Se concentrations in the up-ground parts, roots, stolons, and tubers of potato will increase with increasing Se supplementation, as reported by *Turakainen* (2007). Moreover, Se concentration declined during the growing time in the aerial parts, roots, and stolons of potato plants, while intensive accumulation took place in immature and mature tubers (*Turakainen*, 2007). In seleniferous soils, there are great changes in plants' capability to uptake Se from these soils. It is worth mentioning that most of the cultivated crop plants have a low level of tolerance to high Se levels and, in general, they contain less than 25 $\mu$g Se $g^{-1}$ DW and are non-accumulators like potato (*Ramady et al.*, 2014). It was found that critical Se concentration in plant tissues, which reduced the yield in the case of Indian mustard, rice, maize, and wheat (in $\mu$g $g^{-1}$ DW), was 105, 77, 42, and 19, when Se amounts (as selenite) were 5, 5, 4, and 10 $\mu$g Se $g^{-1}$ soil respectively (*Rani et al.*, 2005).

There are a number of physiological functions or roles of Se in higher plants (*Pilon-Smits & Colin*, 2010; *Hasanuzzaman et al.*, 2010; *Hajiboland*, 2012; *Ramady et al.*, 2014). One of the beneficial effects of Se in plants exposed to stress situations is increasing antioxidant activity. It is reported that plants treated with selenate induced higher increases in plant enzymes that detoxify $H_2O_2$, especially both ascorbate peroxidase and glutathione peroxidase. Application

of low rates of selenate is used to increase the induction of plant antioxidation system and then to facilitate stress resistance, as indicated by *Hasanuzzaman et al.* (2010). An excess of Se may reduce germination and growth rates of non-tolerant plant species and bring about chlorotic leaves and black spots. It is reported that the critical Se concentration in solid media for gain reed (*Arundo donax* L.) plant ranged from 20 to 50 mg $kg^{-1}$ for the American and Hungarian ecotypes respectively (*El-Ramady et al.*, 2014 b). In some plants, increased Se levels repress the concentrations of N, S, and P, just as several amino acids (*Kabata-Pendias*, 2011; *Ramady et al.*, 2014).

Blockage uptake of some metals (mainly Cu, Cd, Mn, and Zn) may happen under high Se concentrations. Hence, the application of N, S, and P is known to help in Se detoxification, which may lead to either supressing the Se uptake by roots or to establishing a healthy ratio of Se for these previous elements (*Kabata-Pendias*, 2011; *Ramady et al.*, 2014).

At a global level, the range of Se in cereals is estimated at 100–800 $\mu g\ kg^{-1}$ FW (*Fordyce*, 2005). This Se range mean (in $\mu g\ kg^{-1}$) varies from 142 to 970 and from 14 to 90 for countries with high and low Se amounts in grains respectively (*Kabata-Pendias & Mukherjee*, 2007). Using soil application of 10 g Se $ha^{-1}$ rate, it is found that Se contents in grains of oats and barley (in $\mu g\ kg^{-1}$) ranged from 19 to 260 and from 32 to 440 respectively (*Gupta & Gupta*, 2000), while using two foliar application rates of Se (10 and 20 g Se $ha^{-1}$) increased Se contents of winter wheat grains from 0.094 to 0.192 mg $kg^{-1}$, and the first Se rate was sufficient for reaching the essential content in wheat grains (*Duscay et al.*, 2006).

A number of feed and forage samples from China were analysed by *Ge & Yang* (1993). They found that these samples were from Se-defecient areas, which contain the following Se levels (in $\mu g\ kg^{-1}$): $< 20$, 30–50, 60–90, and $> 100$ for intensely deficient, deficient, moderately deficient, and adequate Se supply areas respectively (*Kabata-Pendias*, 2011). Thus, the agronomic biofortification with Se-supplemented fertilizers is a usual practice with cereal crops to increase the Se content and nutritional quality of grains (*Banuelos et al.*, 2005). However, the transformation of Se by bacteria and the efficacy of these bacteria regarding Se availability for plants are still not clarified (*Acuna et al.*, 2013).

There are several articles and books concerning the relationship between Se and plant-based foods and human health (*Combs*, 2005; *Hartikainen*, 2005; *Reilly*, 2006; *Rayman et al.*, 2008; *Fairweather-Tait et al.*, 2010, 2011; WHO, 2011; *Banuelos et al.*, 2014; *Ramady et al.*, 2014). In general, people can obtain their Se requirements almost entirely from foodstuffs, whereas Se often

occurs bound to proteins in both plant and animal tissues (WHO, 2011). Hence, seafood, meat, and cereals are considered to be the most significant food sources of Se because they have elevated protein content (for seafood and meat: 0.3–0.5 mg Se $kg^{-1}$) due to their consumption in great amounts (for cereals 0.1–10 mg Se $kg^{-1}$). Fruits and vegetables (foods with almost low protein levels) tend to have low Se content ($< 0.01$ mg Se $kg^{-1}$).

Generally, Se content in different food systems depends on and reflects the soil-available Se to produce those food systems (WHO, 2011). Global Se intakes (in $\mu$g $day^{-1}$) show significant changes among various countries, whereas the average intakes are usually low (10–20), temperate (40–90), and sufficient (85–150) in parts of China, North America, and Europe respectively (FAO/WHO, 1998; WHO, 2011). In more details, it is found that daily dietary Se intake ranges from 7 to 4.990 $\mu$g, with mean values of 40 $\mu$g in Europe and 93 to 134 $\mu$g for women and men in the USA respectively (*Rayman*, 2012). Finally, it is suggested that the average daily Se intake is 53 and 60 $\mu$g for women and men respectively (*Rayman*, 2004).

In the UK, it is reported that the main food groups providing Se in the diet or the contribution of each food group to total population dietary exposure include eggs (4%), vegetables and fruits (7%), fish (10%), milk or dairy products (21%), cereals and bread (26%), and meat (26%). On the other hand, certain types of nuts grown in Brazil are good sources of Se, with concentrations ranging from $\sim$0.03 to 512 mg $kg^{-1}$ fresh weight (*Rayman et al.*, 2008; *Ramady et al.*, 2014). It is reported that Se concentrations in the heart, liver, and kidney of beef tissues are 0.55, 0.93, and 4.5 mg $kg^{-1}$ respectively, whereas values for muscle tissue were around 0.2 mg $kg^{-1}$. *Juniper et al.* (2008) found that supplementation of cattle with Se-enriched yeast increased muscle Se concentration up to $\sim$0.6 mg $kg^{-1}$, while the average Se content in chicken was $\sim$0.2 mg $kg^{-1}$ and in beef $\sim$0.25-0.3 mg $kg^{-1}$ – in the USA (*Fairweather-Tait et al.*, 2011). The total Se content in fish is between 0.1 and $\sim$5.0 mg $kg^{-1}$ (*Fairweather-Tait et al.*, 2010), but the Se content in some marine fish is considered relatively high for shark, cod, and canned tuna ($\sim$1.5, 2.0, and 5.6 mg $kg^{-1}$ resp.; *Reyes et al.*, 2009).

It is worth mentioning that the main Se species in fish contain selenite/selenate (12–45%) and selenomethionine (29–70%), which depend on both the fish species and the whole Se content (*Rayman et al.*, 2008; *Fairweather-Tait et al.*, 2010; *Ramady et al.*, 2014). *Lipiec et al.* (2010) found that eggs of hens contain from 3 to 25 mg Se in every whole egg, whereas Se supplementation in the diet of hens may increase the Se content of eggs up to 0.34–0.58 mg $kg^{-1}$. Se-enriched eggs are widely produced in the entire world (*Fisinin et al.*,

2009). Eggs are sources of the major Se species containing selenomethionine, selenocysteine, and maybe selenite, where the predominant species (>50%) contain selenomethionine in the white of egg and selenocysteine in the yolk of egg (*Lipiec et al.*, 2010). Selenite and selenocysteine are the predominant Se species in cow's milk; moreover, the supplementation plan of dairy cows with Se-enriched yeast is already in use, and after applying this supplementation the main species will contain selenite, selenocysteine, and selenomethionine (*Muniz-Naveiro et al.*, 2007).

It has been found that both fruits and vegetables contain almost low Se amounts. In the case of unenriched vegetables with low Se levels, the main species contain selenite (4%), Se-methyl-selenocysteine (12%), $\gamma$-glutamyl-Se-methyl-selenocysteine (31%), and selenomethionine (53%) in garlic with a natural Se amount of 0.5 mg $kg^{-1}$ (*Kotrebai et al.*, 2000). However, certain vegetables, such as broccoli, onion, and garlic, when grown in Se-rich soil, can accumulate Se, resulting in Se enrichment from <0.5 up to 140–300 mg $kg^{-1}$. The main Se species in Se-enriched foods like onion is $\gamma$-glutamyl-Se-methyl-selenocysteine (63%), selenate (10%), selenomethionine (5%), and several other species (*Hurst et al.*, 2010). We may conclude that the Se species index in vegetables, such as garlic, broccoli, and onion, is different depending on the whole Se level of enrichment, the form of Se used for this enrichment, and the type of the vegetable. Se-methyl-selenocysteine or $\gamma$-glutamyl-Se-methyl-selenocysteine is the predominant species in Se-enriched vegetables. These forms of Se in plant foods have received attention due to purported protection against cancer in animal models when compared with other forms of this element (*Fairweather-Tait et al.*, 2011).

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# Effect of different levels of nitrogen on the total polyphenol and total flavonoid content of sorghum and millet flours

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**Abstract.** Cereals are the most important food sources worldwide. Nowadays, there is an increase in the interest for sorghum and millet grains due to their nutritional quality and health benefits. Our aim was to determine the total polyphenol (TPC) and total flavonoid content (TFC) of sorghum and millet flours, influenced with different levels of nitrogen. The TPC of flours varied between 38.45 and 375.80 mg GAE/100g of the selected cereal flours. The TFC ranged from 106.26 to 117.93 mg CE/100g in sorghum and millet flours.

**Keywords and phrases:** polyphenol, flavonoid, cereal flours, fertilizer

# 1 Introduction

Polyphenols have beneficial effects on the human health due to their antioxidant activity. They can remove free (mainly oxygen) radicals, inhibit the propagation of oxidation, and act as metal chelators (*Alka et al.*, 2013; *Shahidi* and *Ambigaipalan*, 2015; *Stratil et al.*, 2007; Pradeep et al., 2015). Flavonoids also have antioxidant properties, and they can act with other antioxidants as synergists. These flavonoid compounds, such as ferulic acid and vanillic acid, play important roles in reducing the risk and occurrence of cardiovascular diseases and cancer, and they also have an important role in the prevention of diabetes (*Alka et al.*, 2013; *Arendt* and *Zannini*, 2013). Phenolic acids are located mainly in the pericarp and the aleurone layer in sorghum grain (*Chandrashekar* and *Satyanarayana*, 2006). The total polyphenol contents (TPC) and total flavonoid contents (TFC) of sorghum and millet are different. The TPC varies from 58 mg/100 g (*Cardoso et al.*, 2014) to 128.9 mg/100 g (*Alka et al.*, 2013) in sorghum flours. Millet contains 29 mg/100 g polyphenolics (wet basis) (*Saleh et al.*, 2013), the TPC in millet flours is 29 mg GAE/100g dw (*Sharma et al.*, 2016), 51.4 mg/100 g (*Chandra et al.*, 2016), 195.3 mg/100 g (*Alka et al.*, 2013). *Upadhyay et al.* (2015) studied the TPC of different kinds of millet flours, and it varied between 80 and 264 mg GAE/100 g on dry matter. *Nambiar et al.* (2012) found that the total phenol content in different varieties of millet flour ranged from 268.5 to 420 mg/100g of dw. *Shobana* and *Malleshi* (2007) measured the TPC in native (265 mg/100 g) and in decorticated (67 mg/100 g) millet. The TFC in sorghum flour is 45.91–58.85 mg/100 g (*Afify et al.*, 2012) and 65 mg QE/100 g, while in millet flour is 55 mg QE/100 g (*Alka et al.*, 2013). The experiment showed that flavonoids and phenolics could play a role in the inhibition of LDL cholesterol, DNA scission, and oxidation of liposome (*Nambiar et al.*, 2011). As known, some anti-nutritional factors (certain phenolic compounds, fibres or phytates) could decrease the zinc and iron bioavailability (*Sade*, 2009), while high tannin content could reduce digestibility and food values (*Osuntogun et al.*, 1989). Depending on localization, the decortication or milling could reduce the amount of these compounds. Our aim was to determine the total polyphenol and total flavonoid content of sorghum and millet flour samples, which were grown using different levels of nitrogen fertilizer.

# 2 Materials and methods

## 2.1 Materials

Two varieties of *Panicum miliaceum* L. (Maxi and Lovászpatonai pirosmagvú) and one hybrid of *Sorghum bicolor* L. Moench (Zádor) were obtained from Institutes for Agricultural Research and Educational Farm, Research Institute of Karcag, University of Debrecen, in Hungary. These crops were treated by 0 (N0), 40 (N1), 80 (N2), 120 (N3), 160 (N4), and 200 (N5) kg/ha nitrogen (N) fertilizer. The sizes of the parcels were 4.5 m × 4.8 m for millet and 4.5 m × 7.8 m for sorghum; each treatments were applied in four repetitions. Grains were milled by laboratory roller mill (*Devisetti et al.*, 2014). The dry matter content of flours was 14% on average. The flour samples were kept in 4 °C until taken for analysis.

## 2.2 Methods

### 2.2.1 Determination of total polyphenolic compounds

Concentration of total phenolic compounds was determined using Folin-Ciocalteu reagent (*Kim et al.*, 2003). The absorbance was read in a spectrophotometer (760 nm). The solution of gallic acid was used for the standard curve from 0 to 100 $\mu$g/ml concentrations. The results were expressed in mg GAE/100 g of flour (*Cardoso et al.*, 2014).

### 2.2.2 Determination of total flavonoid content

Total flavonoid content was determined by spectrophotometric method (*Meda et al.*, 2005). The absorbance was measured at 510 nm. The standard curve was constructed between 0 and 100 $\mu$g/ml concentrations. The results were expressed in mg CE/100 g of flour (CE = Catechin Equivalent) (*Alka et al.*, 2013).

The statistical analyses based on the ANOVA was carried out using Least Significant Difference ($LSD_{5\%}$) value by *Sváb* (1981).

# 3 Results and discussions

## 3.1 Total polyphenolic compounds

The concentrations of total phenolic compound varied between 38.45 and 375.80 mg GAE/100 g of selected cereals (*Table 1*). The sorghum hybrid flour "Zádor" showed the highest content of polyphenol, from 319.76 to 375.80 mg

GAE/100 g (*Table 1*, *Figure 1*). *Panicum miliaceum* var Maxi showed the lowest TPC content, 53.82–61.27 mg GAE/100 g and *Panicum miliaceum* var Lovászpatonai pirosmagvú showed the lowest content of polyphenol (38.45–53.75 mg GAE/100 g) (*Table 1*, *Figure 2*).

Table 1: Total polyphenolic compounds (TPC) of cereal flours (mg GAE/100 g), fresh weight

| Fertilizer dose kg/ha | Cereal flours[a] | | |
|---|---|---|---|
| | Sorghum[b] | Millet[c] | |
| | Zádor | Maxi | Lovászpatonai pirosmagvú |
| N0 | 346.41 ± 12.3 | 54.68 ± 3.0 | 49.69 ± 2.9 |
| N1 | 339.81 ± 5.8 | 56.34 ± 5.3 | 49.82 ± 1.4 |
| N2 | 319.76 ± 4.7 | 56.25 ± 3.3 | 53.75 ± 6.2 |
| N3 | 346.66 ± 2.8 | 53.82 ± 7.4 | 43.22 ± 4.5 |
| N4 | 350.62 ± 4.1 | 59.12 ± 7.1 | 38.45 ± 1.7 |
| N5 | 375.80 ± 8.0 | 61.27 ± 9.8 | 40.23 ± 1.9 |

a: Results are the mean ± standard deviation of four repetitions
b: $LSD_{5\%}$ between N levels 11.43
c: $LSD_{5\%}$ between N levels 5.03, $LSD_{5\%}$ between varieties 3.18, $LSD_{5\%}$ between N levels*varieties 7.11

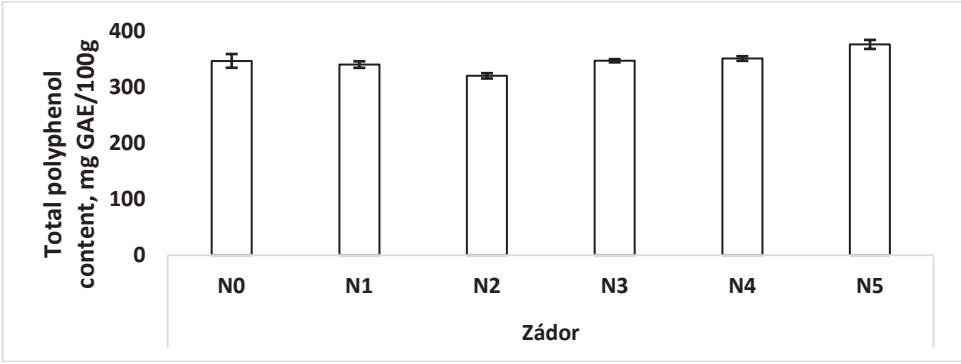


Figure 1: Total polyphenol content of sorghum flours

Significant differences were found in TPC values of "Zádor" flour samples due to the influence of various nitrogen levels ($LSD_{5\%}$:11.43). The differences between influenced millet flour samples could not reach a significant level

($LSD_{5\%}$:5.03). The differences between millet flour varieties ($LSD_{5\%}$:3.18), interaction of nitrogen level and varieties ($LSD_{5\%}$:7.11) was significant on the TPC. The TPC increased with N3-, N4-, and N5-treated samples as compared with control (N0) flour in sorghum samples (*Table 1*, *Figure 1*).

The TPC increased with increasing nitrogen level in Maxi flours, except in the sample of N3 fertilizer dose. The TPC increased only in the case of N1 and N2 treatments in comparison to the control sample (*Table 1*, *Figure 2*).

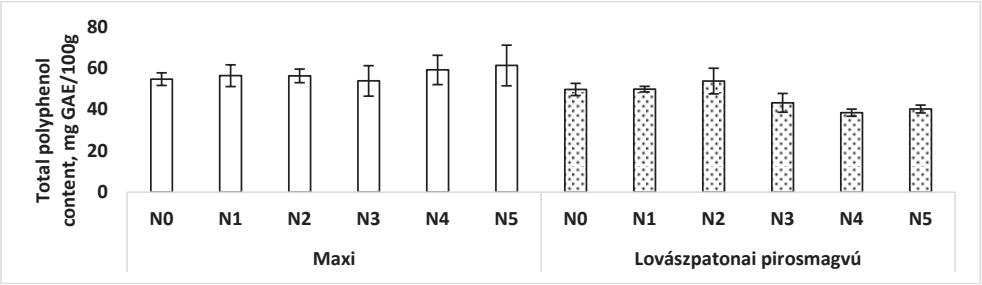


Figure 2: Total polyphenol content of millet flours

## 3.2 Total flavonoid content (TFC)

The highest TFC was showed by "Zádor" flour samples, ranging from 106.26 to 117.93 mg CE/100 g (*Table 2*, *Figure 3*).

Total flavonoid contents in varieties of millet flour samples were similar and varied from 10.26 to 11.46 mg CE/100 g (*Table 2*, *Figure 4*).

Significant differences in TFC values were found in the case of "Zádor" hybrid flours grown with different nitrogen fertilization ($LSD_{5\%}$:6.28). The differences between nitrogen levels ($LSD_{5\%}$:0.61), varieties ($LSD_{5\%}$:0.39), and interaction between nitrogen level and varieties ($LSD_{5\%}$:0.87) were not significant in millet flour samples.

The TFC decreased in "Zádor" flour samples, except in the flour of N2-fertilized sample, where it was higher than the control sample (*Table 2*, *Figure 3*).

Table 2: Total flavonoid content of cereal flours (mg CE/100 g), fresh weight

| Fertilizer dose kg/ha | Cereal flours[a] | | |
|---|---|---|---|
| | Sorghum[b] | Millet[c] | |
| | Zádor | Maxi | Lovászpatonai pirosmagvú |
| N0 | $117.33 \pm 3.1$ | $10.60 \pm 0.4$ | $11.24 \pm 0.3$ |
| N1 | $114.87 \pm 3.0$ | $11.14 \pm 0.6$ | $11.46 \pm 0.5$ |
| N2 | $117.93 \pm 6.4$ | $10.85 \pm 0.7$ | $10.39 \pm 0.7$ |
| N3 | $110.10 \pm 7.0$ | $10.89 \pm 0.1$ | $10.26 \pm 0.9$ |
| N4 | $106.26 \pm 2.9$ | $10.87 \pm 0.2$ | $10.89 \pm 0.9$ |
| N5 | $110.10 \pm 2.8$ | $10.47 \pm 0.5$ | $10.40 \pm 0.7$ |

a: Results are the mean ± standard deviation of four repetitions
b: $LSD_{5\%}$ between N levels 6.28
c: $LSD_{5\%}$ between N levels 0.61, $LSD_{5\%}$ between varieties 0.39, $LSD_{5\%}$ between N levels*varieties 0.87

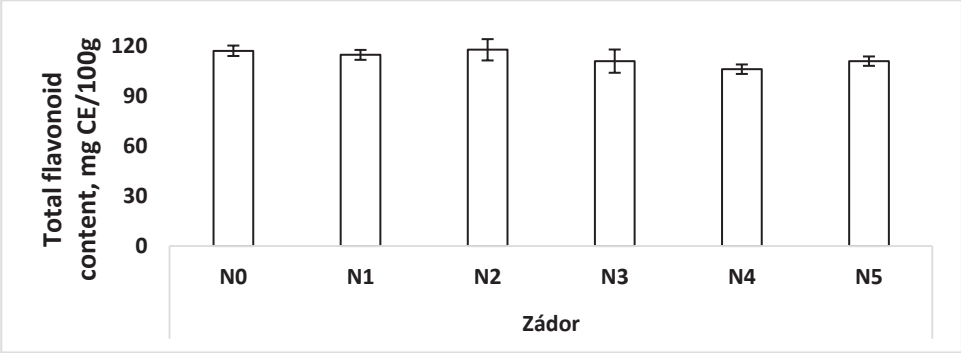


Figure 3: Total flavonoid content of sorghum flours

The TFC was approximately equal, except for N1-treated samples, where it was higher than control in both millet flours (*Table 2*, *Figure 4*).

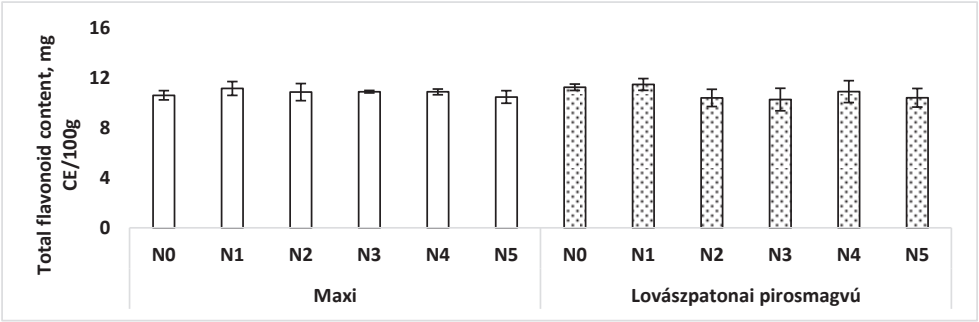


Figure 4: Total flavonoid content of millet flours

## 4 Conclusions

In our experiment, we measured the total polyphenol and total flavonoid content of sorghum and millet flour samples treated with different levels of nitrogen. Sorghum flours have higher TPC and TFC compared to the millet flour samples. The nitrogen fertilizer dose influenced the TPC values of sorghum flours (N3, N4, N5); they increased with the increasing of nitrogen level. The interactions between the influenced samples require further studying.

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# Effects of pectolytic enzyme treatment and microfiltration on antioxidant components of elderberry juice

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**Abstract.** In this study, pectolytic enzymes (*Pectinex BE XXL*, *Trenolin Rot*, and *Fructozym P*) were investigated for their influence on phenolic, anthocyanin content, and antioxidant activities of elderberry (*Sambucus nigra* L.) pulps during juice processing. Prior to pressing the berries,

**Keywords and phrases:** sambucus nigra, elderberry, antioxidant, enzyme treatment, microfiltration

three different enzymes were added to pulps in order to evaluate the effect of different pectolytic enzyme treatments on the valuable components of elderberry juice. Control sample was prepared without enzyme. After treatment, squeezing, and clarification steps, microfiltration was carried out with ceramic membrane. The effect of this technology on the antioxidant capacity, total polyphenol content, and total anthocyanin content of the clarified elderberry juices has been evaluated in permeate and retentate samples, and membrane retention was calculated. Significantly lower antioxidant capacity was detected in the case of control sample than that obtained using enzyme-treated juices. Retention of antioxidant content on the microfiltration membrane was greatly reduced by using the enzymes. Higher valuable component yield was obtained using *Fructozym P* enzyme compared with *Pectinex BE XXL* used in industry.

Elderberry (*Sambucus nigra* L.) is a genus of flowering plants (*Angiospermatophyta*), order of Dipsacales from Adoxaceae family (*Borhidi*, 1995; *Atkinson & Atkinson*, 2002).

In contrast to the well-founded commercial products, the *Sambucus nigra* species gets unreasonably low attention as a herb. This fruit is a rich source of organic acids (malic acid, acetic acid, etc.), vitamins (A, C), microelements, amino acids, sugars, and also essential oils (*Bernáth*, 2000; *Stégerné*, 2010). *Sidor* and *Gramza-Michałowska* (2014) established that elderberry contains several high bioavailable antioxidant components: mainly polyphenols, anthocyanins, flavonols, phenol acids, and anthocyanidins.

Elderberry is consumed only in processed form. Therefore, food producers aim to preserve the biologically valuable molecules during food processing. Next to the beneficial compounds of berries, their natural colorant content accounts for the wide consumption of elderberry. Semi-finished elderberry products are produced in two ways: fruit concentrate and pulp to make common beverages, juices, syrups, jelly, and jam from them. During processing, a filtration technology is applied to clarify the juice, but this step may decrease the stability of valuable components (*Stégerné*, 2010).

One step of technological line of juice production is to break down the pectin molecules with pectolytic enzymes in order to release the beneficial molecules; so, the pressing technology becomes easier and more effective by enzyme treatment. Pectin, a heteropolysaccharide, is contained in the cell walls of all higher plants. In food industry, the main aim of using pectolytic enzymes is to stabilize quality and simplify technological processing (*Barta & Körmendy*, 2007).

Our aim was to investigate the influence of pectolytic enzyme (*Pectinex BE XXL*, *Trenolin Rot*, and *Fructozym P*) on the phenolic, anthocyanin content, and antioxidant activities of elderberry (*Sambucus nigra* L.) pulps during juice processing.

# 1 Materials and methods

## 1.1 Pre-treatment and elderberry juice production

The berries of elderberry (*Sambucus nigra* L.) cultivars "Haschberg" were collected from Nagyvenyim horticultural plant in Hungary. The elderberry juice was prepared by enzyme treatment, pressing, and via microfiltration (MF) steps. The preparations of elderberry juice were performed according to the industrial practice in a pilot plant of Szent István University.

After washing and crushing steps, the pulp was heated at 80 °C in order to inactivate the enzymes of the fruit. The next step was the enzyme treatment; so, the pulp was cooled down to the optimum degree of enzymes (35 °C). The effect of enzymes was studied during the experiments. Before pressing, the berries were treated with three different pectolytic enzymes (*Pectinex BE XXL*, which is used in industry, *Trenolin Rot*, and *Fructozym P*) and one sample was prepared without any enzyme. The enzymes were added to the crushed fruit and left to stand for 1 hour. The amount of enzyme depends on the applied enzyme and according to the industrial practice (*Table 1*). After the first treatment, the pulps were squeezed by manual pressing, followed by a second enzyme treatment with half the amount of the previously applied enzymes for 30 minutes to improve the efficiency of the pressing operation to facilitate microfiltration.

Table 1: Parameters of enzyme treatment

| Enzyme | 1st Enzyme treatment (ml/kg) | 2nd Enzyme treatment (ml/kg) | Distributed by |
|---|---|---|---|
| Pectinex BE XXL | 0.2 | 0.1 | Kerttrade Ltd. |
| Fructozym P | 0.06 | 0.03 | Kerttrade Ltd. |
| Trenolin Rot | 0.02 | 0.01 | Kerttrade Ltd. |

After the enzyme treatments, the clarification experiments were carried out with Klar Sol Super (0.35 mg/l), the samples were left to stand for 20 minutes and then with ErbiGel (0.1 mg/l) for 10 minutes.

The last step of juice preparation was filtration. Clarification experiments were carried out in the laboratorial microfiltration (MF) unit, whose scheme is shown in *Fig. 1.*

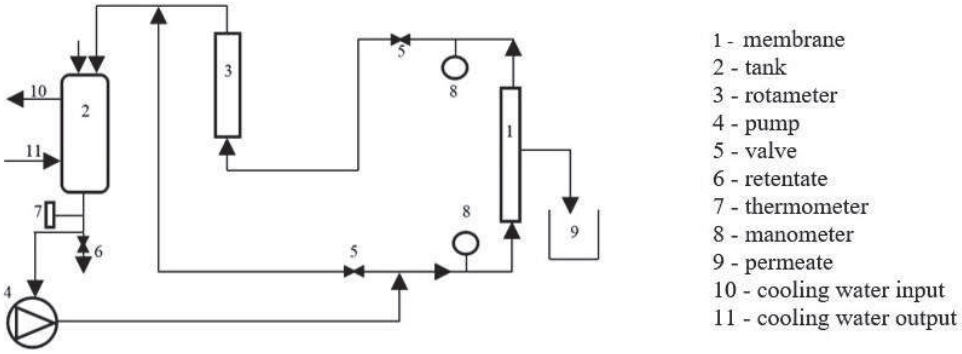


Figure 1: Scheme of the microfiltration unit

The filtration of enzyme-treated juice was applied to ceramic tube membrane (Pall Schumasiv) with an average pore size of 0.8 $\mu$m and a permeable area of 0.005 $m^2$. Inside the tube membrane, a static mixer was used to enhance the permeate flux.

The enzyme-treated juice was recirculated by a pump to the membrane surface, where a cross-flow system provided the reduced amount of fouling layer. The retentate (6. point) was recirculated to the feed tank, while the permeate (9. point) was collected in the beaker. Under the microfiltration process, the flow rate was constant at 150 l/h, the applied transmembrane pressure was 3.5 bar, and the temperature was 30 °C.

## 1.2 Antioxidant measurements

Antioxidant capacity was determined by the FRAP (ferric reducing antioxidant power) assay (*Benzie & Strain*, 1996). Ferric to ferrous ion reduction at low pH causes a coloured complex to form, and the absorbance change was detected at $\lambda = 593$ nm. The results are expressed in ascorbic acid equivalent per litre (mg AAE/l).

The total polyphenol content (TPC) was measured with Folin-Ciocalteu reagents at $\lambda = 765$ nm, using spectrophotometer. The calibration curve was made from gallic acid, using the Singleton and Rossi (1965) method. The results were shown in mg gallic acid equivalent per litre (mg GAE/l).

The total anthocyanin content of samples was determined by pH differential method (*Lee et al.*, 2005) based on the anthocyanin structural transformation, which occurs with a change in pH (coloured at pH 1.0 and colourless at pH 4.5). The change of colour was detected at $\lambda = 520$ nm. The results were expressed in mg per litre (mg/l).

The value of antioxidant capacity (FRAP), total polyphenol content (TPC), and total anthocyanin content (TAC) were calculated to the same dry matter content (it was measured by ATAGO pal- $\alpha$ digital refractometer).

### 1.3 Statistical analyses

Statistical analysis was carried out with the help of Microsoft Excel 2013. ANOVA and Student t-test were used to determine the differences between the resistances of four enzyme-treated samples during microfiltration and also to decide whether enzymes caused significant changes in antioxidant content. The P value $< 0.05$ was considered to be significant.

## 2 Results and discussion

*Permeate flux*

During the microfiltration process, the suspended solids were removed from the elderberry juice. The MF of elderberry pulps was performed until we obtained 200 ml permeate. Time and dry matter content were measured for every 10 ml of permeate. After collecting 200 ml of permeate, we removed it from the tank. The retentate, permeate, and the samples before filtration were kept under refrigeration at -18 °C until subjected to analytical measurements. The permeate flux of different enzyme-treated samples was calculated with the next equation:

$$J = \frac{V}{A \cdot t}$$

J – flux [l/m$^2$h],
V – volume [l],
A – membrane surface [m$^2$],
t – time [h].

The effect of enzyme treatments on permeate flux is shown in *Figure 2*. In all the cases, the typical behaviour of the microfiltration process curve was observed. After a sharp initial flux, it declined due to membrane polarization,

and flux stabilization occurred. According to data, the enzymes influenced the clarification process as well as the permeate flux and the filtration time.

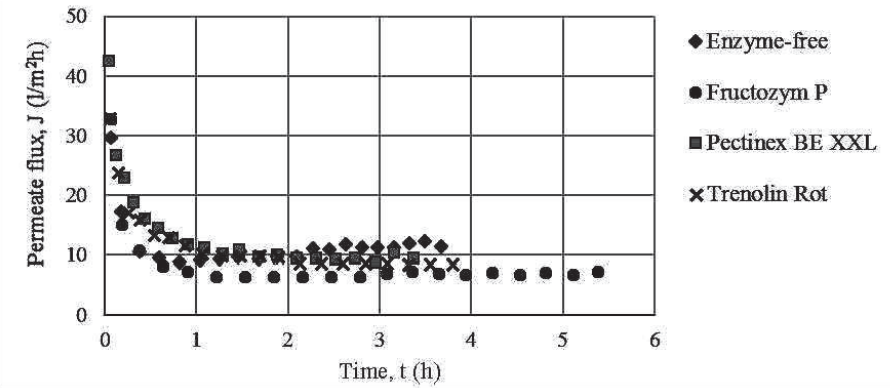


Figure 2: Effect of enzyme treatment on permeate flux during microfiltration

Comparing the values of initial flux, the best result (42.6 l/m$^2$h) was obtained when using *Pectinex BE XXL* enzyme, which is used in food industry, whereas the lowest result was 29.6 l/m$^2$h, obtained at the flux value of the enzyme-free pulp.

Considering the steady flux values, *Pectinex BE XXL* and *Trenolin Rot* enzymes were the same, around 10 l/m$^2$h, while the enzyme-free pulp was 11 l/m$^2$h and the steady flux of Fructozym P enzyme-treated juice was the lowest, around 6 l/m$^2$h; therefore, using this enzyme treatment becomes uneconomical according to the other pre-treatment.

**Results of antioxidant measurements**

After the MF, the antioxidant quality of elderberry juices was measured by spectrophotometric methods. Differences were evaluated between antioxidant capacity, polyphenol, and anthocyanin content of pre-microfiltrated raw juice, permeate, and retentate samples in order to influence the enzyme treatments, and MF were investigated.

*Figure 3* shows the antioxidant capacity of samples. The FRAP values were between 21.76 mg AAE/l and 51.50 mg AAE/l. The clarified enzyme-free elderberry pulps presented the lowest antioxidant content compared to enzyme-treated samples, while the most efficient antioxidant extraction was observed using *Fructozym P* enzyme. Considering the raw-enzyme-treated juices, the lowest FRAP value was measured using *Trenolin Rot* enzyme, which

is probably due to its lower pectolytic efficiency.

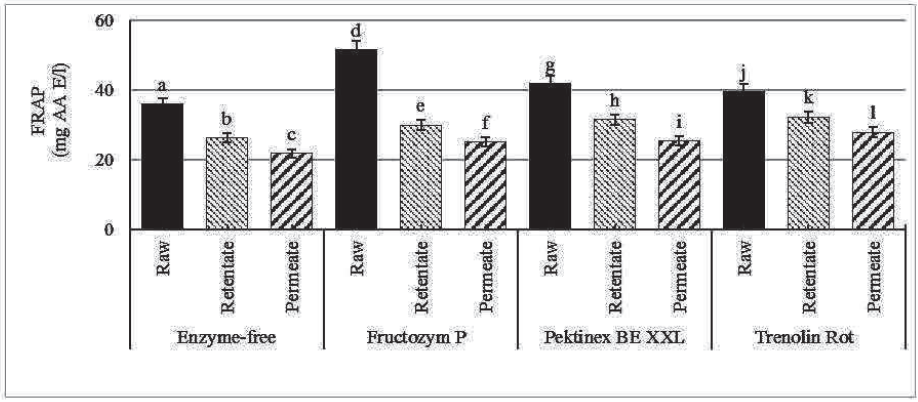


Figure 3: Average results of FRAP (data shown in mg AAE/l)

a, b, c, etc. – the different letters significantly differ at $P < 0.05$

The concentration of antioxidant capacity decreased during the microfiltration process; in the case of all enzyme-treated samples, the FRAP values in the permeate samples were higher than in the retentate.

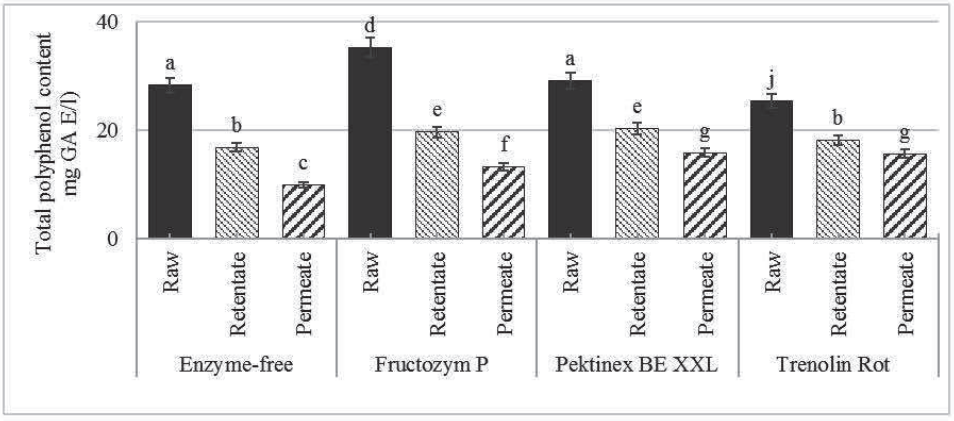


Figure 4: Average results of total polyphenol content (data shown in mg GAE/l)

a, b, c, etc. – the different letters significantly differ at $P < 0.05$

The total polyphenol content of pulps is shown in gallic acid equivalent in *Figure 4*. Similarly, regarding the antioxidant capacity results and their

changes: more polyphenol component was extracted by the enzyme treatment due to the pectin breakdown, except when using *Trenolin Rot* enzyme. The most efficient extraction was experienced with *Fructozym P* enzyme-treated samples, where a significant difference ($p_{\text{value}} > 0.05$) was detected. The polyphenol content of the retentate was 50–70% of the raw juice, wherefore the TPC values of permeate were even lower.

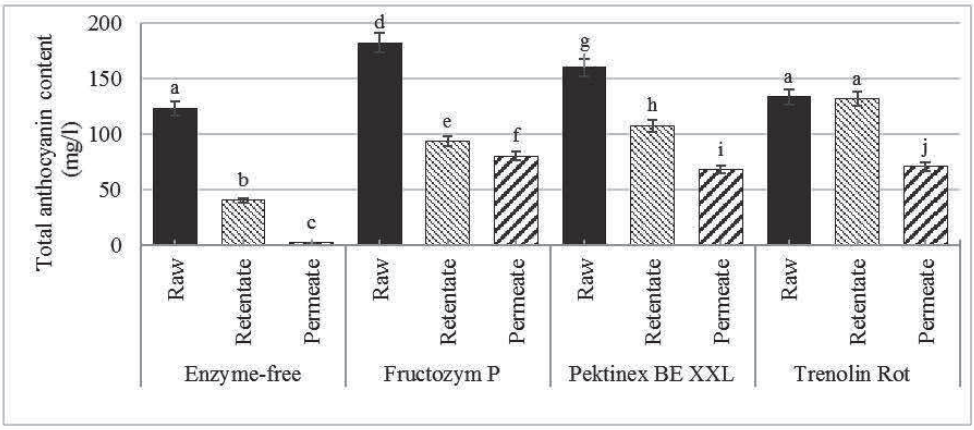


Figure 5: Average results of total anthocyanin content (data shown in mg/l)
a, b, c, etc. – the different letters significantly differ at $P < 0.05$

The total anthocyanin content of elderberry juice samples is shown in *Figure 5*. The anthocyanin content ranged from 1.74 to 182.43 mg/l. The highest TAC of pre-microfiltrated raw pulps was again in the case of the *Fructozym P* enzyme-treated sample, wherefore the lowest was found with the enzyme-free pulp, which had 30% lower anthocyanin content than in the case of using *Fructozym P* enzyme. Due to filtration, the highest reduction of TAC values was detected with the enzyme-free sample – the decrease was 98.6% in the case of the permeate and 67.4% in the case of the retentate.

*Retention of membrane*

The membrane of the microfiltration unit eliminates the suspended components to a varying degree. The rate of retention was calculated with the following equation:

$$R = \left(1 - \frac{C_P}{C_R}\right) * 100$$

R is retention [%], $C_P$ is permeate concentration, and $C_R$ is retentate concen-

tration.

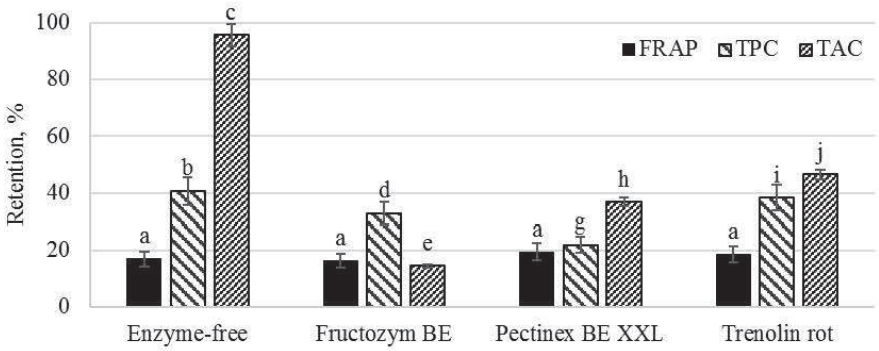


Figure 6: Retention rate of antioxidant components on the MF membrane

FRAP: antioxidant capacity, TPC: total polyphenol content; TAC: total anthocyanin content
a, b, c, etc. – the different letters significantly differ at $P < 0.05$

*Figure 6* shows the retention rate of antioxidant molecules. The membrane retained antioxidant components at the highest rate in the case of the sample prepared without enzyme treatment. Then enzyme treatment was carried out, and the retention of membrane significantly decreased. Considering the retention of antioxidant capacity and polyphenol content in the case of *Trenolin Rot* enzyme-treated samples, approximately the same retention rate was obtained as with enzyme-free samples. This retention rate was significantly higher than in the case of using *Fructozym P* and *Pectinex BE XXL* enzymes.

## 3 Conclusions

The aim of this study was to evaluate the effects of pectolytic enzyme treatment (*Pectinex BE XXL*, *Trenolin Rot*, and *Fructozym P*) in the course of elderberry juice microfiltration and to observe how it affects the retention of the components responsible for antioxidant capacity.

The results of raw elderberry pulp measurements show that the enzyme treatments resulted in higher antioxidant components than the enzyme-free sample. Using *Fructozym P* enzyme, pectin breakdown was reached more efficiently compared to *Pectinex BE XXL*, which is used in industry. The highest antioxidant capacity, polyphenol and anthocyanin content were determined in this pulp, although the time of MF also increased in this case.

The lowest valuable components were measured using *Trenolin Rot* enzyme, where the results were statistically equal to the enzyme-free juice values.

Significant losses are believed to have occurred after the MF clarification process due to fouling of membrane pores, which can be decreased with pectolytic enzyme treatment. The most efficient filtration took place with the use of *Fructozym P* and *Pectinex BE XXL* enzymes.

Research results of *Yu* and *Lencki* (2004) are the same: according to the microfiltration of the enzyme-treated apple juice, more reduced antioxidant capacity, increased flux value, and lower resistance were detected than in the case of using enzyme-free pulp.

## Acknowledgements

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# The role of vitamins in the diet of the elderly I. Fat-soluble vitamins

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**Abstract.** Following a discussion on the daily energy and protein requirements of elderly people, the authors will go on to talk about vitamin needs and the role of the four fat-soluble vitamins (A, D, E, and K). They point out that vitamin requirements in old age do not essentially differ from adult people's, but they must take account of the fact that the body's vitamin stores might get filled up, which may reduce vitamin needs, on the one part, but the altered physiological processes may increase them, on the other. Regarding the case of fat-soluble vitamins, reduced fat absorption, decreased vitamin storage capacity of the liver, reduced dietary intake, partial deficiency of digestive enzymes, and absorption disorders in the intestines may all lead to vitamin deficiencies. Problems may also arise due to multiple vitamin overdose developed either as a consequence of overconsumption of vitamin tablets or because

**Keywords and phrases:** vitamin A, vitamin, D, vitamin E, vitamin K, fat-soluble vitamins, vitamin needs, vitamin requirements of the elderly

the body's vitamin stores are constantly filled up to maximum capacity. Positive and negative changes resulting from the consumption of several times the daily dose recommendations are covered as well. The authors show that A, D, E, or K vitamin deficiency occurs very rarely in the case of a normal diet; however, great care must be taken in order to meet vitamin D and, simultaneously, calcium requirements so that to avoid osteoporosis and an increased risk of bone fractures in elderly people. The paper discusses the fat-soluble vitamin needs of the elderly and, where necessary, specifies the requirements for men and women separately, while also touching upon those foodstuffs and methods that can contribute to the optimal satisfaction of the elderly people's vitamin needs.

# 1 Introduction

In order for our organism to work properly, we need a regular daily consumption of foodstuffs through which we can get access to vital proteins and energy. As the age increases, especially after 40 years of age, people's energy needs are on the decline (*Hallfrisch et al.*, 1990), and an older person can easily make do with one third less of energy (9,000–9,500 kJ/day) than a forty-year-old does (11,000–11,500 kJ/day). In general, we can say that an approx. 70-year-old person can get along with 30% less energy than a younger individual and that the daily energy intake of 60–80% of elderly people living in a community is 4,000–4,500 kJ (*Mcgandy et al.*, 1966; *Abraham et al.*, 1977). The second half of the past century saw a decrease of 4,000–5,000 kJ/day and of 3,000 kJ/day in the energy intake of men and women, respectively, which entails a reduced intake of several important nutrients (*Thomas*, 2007).

The biggest problem lies in the decreased protein intake, since 10% of men and 20% of women consume proteins below the level prescribed by the RDA (Recommended Dietary Allowance). Less protein intake means less mineral and vitamin absorption, leading to various deficiency diseases (*Wakimoto & Block*, 2001). The vitamin and mineral intake in 50% of the elderly people does not reach the necessary values specified in the RDA, and thus 10–30% of the respective population suffers from deficiencies. Elderly people tend to consume less foodstuff rich in calories and they rather resort to calorie-restricted diets consisting of whole grains, vegetables, and fruits, in parallel to which both the amount of dietary intake and fluid consumption are on the decrease. On top of this, all those physiological changes that accompany the aging process, such as slower emptying of the stomach, altered senses of taste and smell, hormonal changes, and mastication difficulties as a result of changes in dentition, make

their contribution to this.

Reduced food and energy intake is also closely related to negative changes in terms of income, the education level of those affected, changes in the family and socio-economic status, as well as opinions, beliefs, and misbeliefs regarding foodstuffs. Consequently, reduced calorie and nutrient intake lead to dietary-related deficiency diseases. First of all, those elderly people are at risk who consume a vitamin-deficient diet, are vegetarians, or suffer from some food-ingredient-related malabsorption. Particular attention should be paid to those suffering from lactose and milk protein intolerance as they cut themselves off from sources of high biological value protein and the highly beneficial milk fat (*Drewnowski & Shultz*, 2001).

In the majority of the world's countries, healthcare authorities determine for all age-groups the necessary minimum values of calories and food ingredients that are indispensable for a healthy lifestyle, expressed in RDA levels. They took account of body weight, stage of development (child–adult), and gender (male–female), but no distinction was made between individuals having the same body weight but belonging to different age-groups. Officially determined RDA levels have gone through continuous changes, but later on they also took into consideration the fact that the human organism is capable of stockpiling certain nutrients that it can put to work in cases of malnutrition. They also established that the long-term effects of foodstuffs consumed for therapeutic purposes in quantities above the average is controversial (*Thomas*, 2007).

In an attempt of doing away with the errors of the RDA, they introduced the DRI (Dietary Reference Intake), which did not only take account of nutrition requirements but it also reckoned with disease prevention. On the 'design table', it was considered a basic requirement that the determined values should fully satisfy the needs of 50% of the population, whereas 98% should not suffer from any deficiency diseases in regard to nutrients without an RDA value. They determined the appropriate intake values and the upper limits that could still be tolerated and did not cause any risk in 98% of the population. RDI includes the senior population over 70 years of age, paying separate attention to men and women. *Table 1* shows the RDI values of fat-soluble vitamins and the tolerable upper limits (*Thomas*, 2007).

If we compared the data provided in the table with values determined for the younger population, only slight differences could be observed.

Table 1: RDI values of fat-soluble vitamins for the male and female population over 70 years of age and the tolerable upper limits

| Vitamin | Men | Women | Upper limit |
|---|---|---|---|
| Vitamin A ($\mu$g) | 900 | 700 | 3,000 |
| Vitamin D ($\mu$g) | 15 | 15 | 2,000 |
| Vitamin E (mg) | 15 | 15 | 1,000 |
| Vitamin K (mg) | 120 | 90 | No data |

## 1.1 Macronutrient requirements

For an optimal bioavailability of vitamins, the human organism needs an appropriate supply of energy, proteins, fat, and carbohydrate. Daily calorie requirements for sedentary activities were determined at 100–110 kJ/kg/day, while the corresponding values were assigned in the range of 160–170 kJ/kg/day for elderly people living under normal conditions. There are different formulae scientists apply in practice to assess energy requirements (*Ireton-Jones*, 1989). Protein requirements are 0.8 g/kg/day (NRC, 1989), but if we wish to establish a positive nitrogen balance in older people, then this value is 1.0–1.2 g/kg/day (*Campbell et al.*, 2001). People more advanced in age have need of proteins because the essential amino acids found therein are the very construction bricks of the proteins and of various, biologically valuable components in their organism. Assuring an optimal supply of energy and proteins is fundamental as, for lack of energy, the organism will not be capable of building the absorbed amino acids into the body, and it will make use of them to obtain more energy.

The optimal carbohydrate intake responsible for 50–60% of the organism's energy requirements is highly essential as a great part of them is utilized as energy source, protecting proteins from degradation and their utilization in gluconeogenesis. An insufficient amount of energy and carbohydrate intake leads to muscle atrophy since proteins are thus utilized as energy, and they will degrade together with subcutaneous tissues. Whole-grain breads, cereals, legumes, vegetables, fruits, and milk all contain a sufficient amount of carbohydrates (*Thomas*, 2007).

Fat is the most energy-dense nutrient, stored in the organism in the form of triglyceride in the adipose tissue. The optimal amount of energy via fat intake is 20–25%, which can be occasionally much higher, resulting in obesity. There are no recommended doses for fat intake, but there is proof that fats with a

balanced fatty acid composition (oil seeds, poultry, fish, and vegetables) are much healthier as they contain a sufficient amount of essential fatty acids as well.

Besides calories and proteins, dietary fibres – originally part of our foodstuffs – are essential components of our foods, just as added fibres, both being indigestible substances but with several beneficial effects. Fibre content is beneficial to health, playing a prominent part in reducing the symptoms of failures of the cardiovascular system and preventing events of stroke and certain types of cancer. A clear association was found between high fibre consumption and a low incidence rate of cardiovascular diseases, in particular in cases when cereals, fruits, or vegetables served as fibre sources, though we also know of such experiments where this relationship was inconclusive (*Wolk et al.*, 1999; *Steffen et al.*, 2003). Several researchers revealed a correlation – while some others did not – between colon cancer and fibre consumption. It has been a well-known fact that as fibres swell up they help the movement of faecal matter, prevent constipation, and on a large area bind excess bile acids as well as other, undesirable substances entering the body (*Park et al.*, 2005). The optimal daily fibre intake for men is 38 g/day and for women 25 g/day. Since fibre content is difficult to digest and it also reduces the bioavailability of other nutrients, over 50 years of age, the optimal amount is 30 g/day for men and 21 g/day for women.

On the whole, there are no significant differences between the DRI values recommended for the macronutrient components in respect of age-groups over 50 years. Recommended daily water intake for men is 3.7 l and for women 2.7 l. Daily carbohydrate intake was determined at 130 g, protein intake (calculated at 0.8 g/kg/day) at 56 g for men and 46 g for women, while fibre intake at 30 g for men and 21 g for women (*Thomas*, 2007).

## 2 Vitamin requirements

For the preservation of human life, we also need certain natural organic compounds in small quantities that play a role in the control of metabolism and energy metabolism as well as in the regeneration of the body. These essential substances are the vitamins. They are such organic compounds that the human body cannot – at least not sufficiently – synthesise, they do not provide energy, but they are indispensable for metabolism and energy flow.

The effect of vitamins is based on the fact that acting as catalytic or regulatory factors in different parts of the organism they get involved in the phys-

iological processes. Some vitamins become bound to proteins, and take part in biochemical processes as enzymes, while another group of them performs a crucial role in protein synthesis. Daily vitamin requirements are dependent on age, state of health, and nature of the work performed. Humans get access to the necessary vitamins by consuming foods of plant and animal origin, while some vitamins can be obtained with the help of microorganisms living in the intestinal tract (*Csapó & Csapó–Kiss*, 2002).

It has been assumed that fat-soluble vitamins regulate the biosynthesis of certain proteins. In their absence, enzyme activity will either decrease or increase in certain tissues. The organism is able to store fat-soluble vitamins, wherefore related cases of avitaminosis are much rarer, but there is a higher risk of hypervitaminosis regarding, first of all, vitamins A and D.

In older people, lower levels of energy and protein intake often go hand in hand with inadequate vitamin supply, especially when combined with insufficient vegetable and fruit consumption. About 50% of the elderly consume vitamins below the prescribed RDA levels, while 10–53% of them hardly reach the desired levels (*Foote et al.*, 2000; *Souba*, 1997), which is also due to the fact that only 10% of them consume vegetables and fruits regularly, on a daily basis. According to a survey, 41% of the elderly people do not consume fruits on a daily basis, while 17% of them do not consume vegetables regularly (*Block*, 1991). Vitamin A deficiency has been observed only occasionally despite a decreased consumption. However, vitamin D deficiency is affecting 3–7% of the elderly population, which in extreme cases, in retirement homes, can reach as high as 35%. As in old age body fat increases, the organism can easily store fat-soluble vitamins, making their daily intake unnecessary.

Nevertheless, the organism does not dispose of such storage levels in terms of water-soluble vitamins, which is why vitamin B – except for vitamin $B_{12}$ stored in the liver – requirements have to be met on a daily basis. Consequently, vitamin deficiencies were observed in 13–43% of the elderly for vitamin $B_1$ and in 5–56% for vitamin $B_6$ (*Hajjar & Pharm*, 2007). Such typical cases of vitamin deficiency diseases as scurvy, beriberi, or pellagra have a very low incidence rate among the senior population. Vitamin deficiencies tend to be symptomatic in cases of insufficient consumption of foods in terms of quantity and quality or when people refuse to consume certain types of food on a whim (*Gordon*, 1993).

The importance of an adequate supply of vitamins is also confirmed by scientific research pointing out the vitamins' efficiency against free radicals responsible for the development of certain diseases and known to be associated with ageing. The idea was initially received with scepticism but after

all accepted, thus foregrounding the consumption of vitamins with antioxidant properties. It has become common practice to consume vitamins with antioxidant properties in doses way above physiological levels in order to prevent diseases and treat chronic diseases. Patients were encouraged to consume large quantities of vitamins as these would help prevent cardiovascular diseases and cancer. Pauling (1993) was one of the pioneers of this theory, supporting the idea that the consumption of large amounts of vitamin C helps in slowing down the ageing process.

Considering the range of vitamin preparations developed in the past few years, elderly people paying due attention to their alimentation do not have to reckon with vitamin deficiency. Therefore, people consuming vitamin-deficient foods can also gain access to vitamin supplies by the regular consumption of vitamin supplements (*Hajjar & Pharm*, 2007).

## 3 Fat-soluble vitamins

Generally speaking, our organism has A, D, E, and K vitamins stored up, which is why in case of malnutrition they will leave our body at a very slow pace. With age, storage levels for fat-soluble vitamins can even reach the threshold values of hypervitaminosis.

### 3.1 Vitamin A

Vitamin A deficiency causes nyctalopia, also known as night-blindness, which gave it the name of 'epithelium-protector' vitamin, necessary for keeping the skin and the mucous membrane intact, protecting the organism from invaders coming through these surfaces. The most severe symptom of vitamin A deficiency is blindness, which occurs consequent upon the drying out of the cornea. In its absence, a reduced immune activity sets in, the white blood cell count decreases, and an increased susceptibility develops to pneumonia and respiratory diseases. In terms of the organism, this is a growth factor since its deficiency slows down the development of bones in young people. Hypervitaminosis leads to loss of hair, inflammation of the skin resulting in peeling, and pain in the extremities.

Plants contain only the provitamins of vitamin A. Vitamin $A_1$ may be formed from approx. 12 types of carotenoid compounds, out of which $\alpha$-, $\beta$-, and $\gamma$-carotene as well as cryptoxanthin are the most important ones. Among our foodstuffs, cod-liver oil, chicken-, pork-, and goose-liver all abound in vitamin A, whereas egg, milk, and dairy products may also have a consider-

able amount of vitamin A in store. Carrot, rhubarb, and spinach are rich in $\beta$-carotene.

Bioavailability of vitamin A, but especially of carotenes, is more efficient in the presence of fats. Upon the consumption of raw vegetables, only about 2% of the carrot's carotene content gets absorbed, while the rest is eliminated along with the faecal matter. In the human organism, carotenoids are enzymatically transformed into vitamin A, and are stored in the liver as vitamin $A_1$ fatty acid esters. They fight off the invasion of free radicals and, acting as excellent antioxidants, protect oxidation-sensitive food ingredients as well as the membranes in our body from oxidation and degradation.

Vitamin A is absorbed through the epithelial cells of the small intestine, and is transported through the lymphatic system to the liver, where the organism stores it up. The liver contains 50–95% of the body's vitamin A content. The vitamin A level of the blood is highly controlled, wherefore it is only marginally affected by vitamin A intake and its level is barely conducive to the vitamin A balance of the organism, given that vitamin A level in the blood is mostly dependent on the extent of the vitamin released from the liver (*Hajjar & Pharm*, 2007).

Contrary to vitamin A, carotenes are absorbed from the small intestine through passive transport, and transformed at first into vitamin A aldehyde in the epithelial cells, and then via reduction into vitamin A alcohol, ending up in the liver, where they are stored up. Apart from the liver, carotenoids can be stored up in human tissues such as adrenal glands and the adipose tissue. Lycopene can be found mostly in the testicles, while the oxygenated carotene derivatives (zeaxanthin, lutein) are mostly located in the macula lutea, or yellow spot, which is free of $\beta$-carotene (*Hajjar & Pharm*, 2007).

The daily vitamin A requirement of humans is 0.8–1.5 mg of vitamin A and 5–9 mg of $\beta$-carotene. Over 50 years of age, this value decreases to 700 $\mu$g/day for men and 900 $\mu$g/day for women. Developed countries register very few cases of deficiency diseases among the elderly population. If it does occur, however, some other reasons may lie behind, such as reduced fat absorption, other anomalies, liver problems (its incapability of storing up vitamin A), decreased dietary intake of vitamin A, or its increased excretion via the kidney (*Hajjar & Pharm*, 2007).

An examination of elderly people aged 65–75 years concluded that with the passing of the years the vitamin A content of the liver does not show any decrease (*Hoppner*, 1968), but the amount of vitamin A released from the 'depository' does decrease, giving rise to the occurrence of toxicity events (*Krasinsky et al.*, 1989). We must take account of hypervitaminosis if our regular, daily

vitamin intake exceeds 3,000 $\mu$g, which is three–four times the recommended value. The malfunctioning of the kidney or liver increases the possibility of toxicity, which can be partly compensated with vitamin E. Hypervitaminosis A in the elderly may cause pain in the bone, osteitis, and hypercalcemia as vitamin A – developing a negative calcium balance – increases vitamin D activity and parathyroid-hormone activity. This decreases bone mineral density and increases predisposition to bone fractures (*Kneissel et al.*, 2005; *Rohde & Deluca*, 2003).

Carotene deficiency is practically inexistent and there is no RDA value in this respect. Carotene is not considered a toxicant, and there is no record ever of a high intake of carotene to cause any sort of disease as well as no proof of a large amount of carotene, transformed into vitamin A, leading to any adverse effects. Nevertheless, overconsumption of carotene is not desirable as it causes people to have a slightly yellowish skin (*Mathews-Roth*, 1986).

## 3.2 Vitamin D

Vitamin D (calciferol) controls the absorption of calcium and phosphor as well as regulates their incorporation into the bones. Sufficient exposure to sunlight in the case of adults makes vitamin D supplementation unnecessary. The various forms of vitamin D are produced from provitamins ergosterol of plant origin (ergocalciferol, vitamin $D_2$) and 7-dehydrocholesterol of animal origin (cholecalciferol, $D_3$), upon exposure to UV radiation. Ergosterol can be found in larger quantities in foodstuffs of plant origin, in yeast, certain mushrooms, and ergot. 7-dehydrocholesterol is a steroidal compound present as a cholesterol accompaniment in the subcutaneous fat.

These two forms have been known to be functioning the same way in the body, but *Armas et al.* (2004) showed that vitamin $D_3$ is three times as effective as $D_2$. Vitamin D from both sources attaches to the vitamin D-binding protein, and, carried together to the liver, is hydroxylated into 25-hydroxy vitamin D (calcidiol), which is further hydroxylated in the kidney into 1.25-dihydroxy vitamin D (calcitriol). Calcitriol is the most active form among them, with a 1,000 times higher affinity to nuclear receptors than calcidiol. The half-life of calcitriol in the body is 4–6 hours, whereas that of the calcidiol is three weeks (*Thomas & Demay*, 2000).

Daily vitamin D requirement up to 20 years of age is 10 $\mu$g, while for adults, except for expectant and nursing mothers, is 5 $\mu$g. An accurate assessment of needs is difficult to make as vitamin is constantly produced in the body, on the skin surface, whenever exposed to sunlight. Large amounts of vitamin D can

be found in caviar, salmon, butter, chicken-, goose-, pork-, and beef-liver. Our daily foodstuffs contain first of all provitamins, the largest amount of which can be found in milk, butter, liver, and egg yolk (*Hajjar & Pharm*, 2007).

Vitamin D deficiency is common among the elderly population and it may cause serious health problems. The most severe issues are related to advanced cases of osteoporosis and an increased probability of bone fractures. Despite that our body can store up vitamin D, as we age, the storage level of both vitamins ($D_2$, $D_3$) decreases in the blood serum. 25% of the elderly population living in retirement homes has shown symptoms of vitamin D deficiency, but this number can go up as high as 80% in cases of long-term stay. Most probably, the lack of exposure to direct sunlight may be held responsible for this, leading to a decrease in the skin's 7-dehydrocholesterol content, of which vitamin D is produced in the organism of young people. As synthesis in the skin is suppressed, the vitamin D content of the foodstuffs gains a crucial importance in terms of supply. This becomes particularly dangerous when the ratio of milk, dairy products, and other foodstuffs having a high vitamin D content decreases in the nutrition of elderly people. Reduced hydroxylation in the liver and kidney as well as a reduced absorption induced by drug treatments can also contribute to vitamin D deficiency.

Consequent upon these, the elderly need more vitamin D compared to the younger population. The optimal daily intake for the 51–70 age-group is 10 $\mu$g, for those over 70 years, this quantity is 15 $\mu$g, while for severely vitamin-deficient individuals 20 $\mu$g is the recommended amount so as to increase bone mineral density and reduce the risk of bone fractures. This level can be easily attained if multivitamin tablets are administered twice a day or with nutritional supplements rich in vitamin D content. Parallel to vitamin D supplementation, it is highly expedient to apply calcium supplementation as well because the nutrition of the elderly rarely reaches the required level (*Kamel & Hajjar*, 2003). Therefore, most vitamin D preparations also contain 200–600 mg of calcium.

The beneficial effect of vitamin D supplementation in the elderly has been demonstrated by way of clinical experiments too. In an 18-month-long experiment, a supplementation of 20 $\mu$g of vitamin D and 1,200 mg of calcium resulted in a significantly reduced probability of hip-bone (43% less) and other bone (32% less) fractures as compared to control. Bone mineral density increased by 2.7% in the thighbone, while this value showed a 4.6% decrease in those treated with placebo (*Chapuy et al.*, 1992). Based on results, it is recommended that risk groups, such as elderly people, take at least 10–15 $\mu$g of vitamin D and 800–1,000 mg of calcium per day for a balanced calcium content

in the organism and for the development of an adequate bone strength. Higher doses of vitamin D supplementation (250–2,500 $\mu$g) per year administered by injection is not recommended by most (*Holick*, 1994).

During an experiment with elderly, frail women, 20 $\mu$g of vitamin D supplementation combined with 1,200 mg of calcium reduced the number of falls by 49% as compared to the group given only calcium supplementation, what could be explained with an improved functioning of the musculoskeletal system (*Gloth et al.*, 1995). Most experiments led to the conclusion that vitamin D supplementation had reduced the number of falls by 20% (*Bischoff-Ferrari et al.*, 2004).

The toxic effect of vitamin D in individuals consuming large quantities thereof over a long period of time can occur in very rare cases. For this to happen, however, people would have to consume 50–100 times the optimal daily dose, which would then lead to hypercalcemia, an increased level of calcium in the serum and urine (*Johnson et al.*, 2002). Excessive sunbathing combined with an optimal vitamin D intake can never lead to vitamin D intoxication.

### 3.3 Vitamin E

The various forms of vitamin E (tocopherols, tocotrienols) are antioxidant compounds in the protection of essential fatty acids and membrane lipids from oxidation. For this effect, the hydroxyl group found on the aromatic ring is responsible. Besides the antioxidant effect, it suppresses the synthesis of the leukotrienes, through which, acting as a lipoxygenase inhibitor, it has an anti-inflammatory action on the body. Its consumption in larger doses modifies the synthesis of prostaglandins, thus inhibiting platelet aggregation. It also has free-radical scavenging capacity, upon which it can regenerate helped by vitamin C, and then take effect repeatedly. Vitamin E reduces vascular permeability and influences collagen formation (*Hajjar & Pharm*, 2007).

As a fat-soluble compound, it is absorbed from the intestines as chylomicron micelles formed by bile acids. It is released in the bloodstream, induced by lipoprotein lipase, and it gets through to the liver with the help of various transport processes – from here, it finds its way back into the bloodstream under the form of very-low-density lipoprotein. 90% of it is stored in the adipose tissue, although cell membranes and the lipoproteins circulating in the bloodstream also have the necessary binding capability.

In human nutrition, $\alpha$-, $\beta$-, $\gamma$-, and a $\delta$-tocopherols have practical relevance (*Csapó & Csapó–Kiss*, 2002). Among them, $\alpha$-tocopherol has the highest biological activity, though $\gamma$-tocopherol is the most wide-spread in foodstuffs.

Therefore, RDA values for vitamin E are expressed in $\alpha$-tocopherol equivalents. Due to stereoisomerism, natural $\alpha$-tocopherol gets absorbed more efficiently and its activity is higher than that of its synthetic counterpart.

The natural occurrence of tocopherols is related to plants alone. They are oils of yellowish colour that dissolve only in fats and degreasing agents. The oxidative effect causes them to become compounds with sensitive and reducing properties; therefore, losing their biological activity, they degrade when exposed to air and direct sunlight. They can be used as antioxidants since they are able to inhibit the autoxidation of fatty acids, wherefore their presence delays the rancidity of fats.

$\gamma$-, and $\delta$-tocopherols have the highest antioxidant effect. Humans' daily vitamin E requirement is 15 mg – it is assumed that the amounts of unsaturated and polyunsaturated fatty acids from dietary intake proportionally increase vitamin E requirements. Especially large quantities of vitamin E can be found in the seeds of legumes, the germ oil of cereal grains, butter, and leafy vegetables. E-avitaminosis or -hypovitaminosis has never been shown in humans.

In old age, vitamin E deficiency would occur only in cases of malabsorption of fats, pancreatic problems, enteritis, or coeliac disease. In these circumstances, vitamin E deficiency can lead to a whole range of aetiological diseases, such as anaemia caused by the increased haemolysis of erythrocytes, slow coagulation, and a multitude of neurological disorders, where vitamin E deficiency is only one of the many causes. Although the daily vitamin E requirement of an adult person is 15 mg, intake values in the case of elderly people may reach up to 400–800 mg per day without being conducive to diseases. Nevertheless, we must be careful with regular cases of overdose as regular intake of daily quantities exceeding 1,000 mg can lead to muscle weakness, exhaustion, nausea, or diarrhoea. High doses may be accompanied by haemophilia if the patient uses medicinal products containing coumarin or other, anticoagulant substances.

### 3.4 Vitamin K

Vitamin K (phylloquinone) deficiency leads to severe haemophilia appearing first of all in the gastrointestinal tract. The bleeding wounds do not heal up and coagulation does not take place because the liver cannot synthesise an adequate amount of prothrombin, the protein necessary for blood clotting. Human intestinal flora synthesises a sufficient amount of vitamin K, wherefore deficiency cannot occur in a healthy organism. In the largest quantities,

it can be found in green leaves, spinach, and cabbage, whereas amongst foods of animal origin the liver contains it in high quantities. Plants contain vitamin $K_1$, or phylloquinone, while intestinal bacteria synthesise vitamin $K_2$, or menaquinone. Both types are necessary for a normal functioning of the body.

The daily vitamin K requirement for an adult person is 100 $\mu$g, which is covered by the daily vitamin K synthesis of the nutrients and intestinal bacteria. A continuous intake is required as the vitamin K reserves of the liver are very low. Vitamin K is needed for prothrombin synthesis and it acts as cofactor of enzymes such as $\gamma$-glutamyl carboxylase. It is required for the binding of calcium, the activation of pro-coagulant factors, the synthesis of proteins regulating bone calcium content, and for a normal osseous metabolism.

Daily vitamin K requirement for women over 50 years of age is 90 $\mu$g and for men is 120 $\mu$g. Irrespective of the fact that the liver is capable of storing only a small amount of vitamin K, as a rule, no deficiencies occur in elderly people in cases of normal, complex nutrition, since foods of both plant and animal origin contain significant amounts of vitamin K. Our organism is capable of recycling vitamin K and a normal intestinal flora will produce it in significant amounts. Deficiencies in elderly individuals can take place only if the body is seriously traumatized, the patient has undergone a major gastrointestinal surgery, antibiotics are taken regularly over a long period of time, fat indigestion occurs, a drug therapy is under way that renders the absorption of vitamin K as well as its mechanism of action difficult, and in cases of malnutrition and starvation. Serum vitamin K measurement does not provide sufficient information on the vitamin supply, especially when the details of vitamin intake are not known. In the senior population, as much as 500 times the RDA value did not cause toxic symptoms.

Vitamin K has been recently associated with the formation of healthy bones since the mineral-binding capacity of osteocalcin is in correlation with the vitamin-K-dependent $\gamma$-carboxylation of glutamic acid molecules. The amount of inadequately carboxylated osteocalcin increases with age, which is closely linked to hip fractures in elderly people (*Szulc et al.*, 1993). Small quantities of vitamin K supplementation reduced the amount of inadequately carboxylated osteocalcin and of the calcium eliminated via urinary excretion (*Vermeer et al.*, 1999). In male and female subjects with an average age of 75 years, small quantities of vitamin K supplementation have been shown to significantly reduce the probability of hip fractures (*Booth et al.*, 2000).

## 4 Conclusions

Elderly people can put vitamins to optimal use only in the conditions of a normal energy and protein intake, as enzymes and energy are required for the release and absorption of vitamins from the nutrients as well as for their transport and the related biochemical processes. We may conclude that in the case of optimally nourished elderly people vitamin requirements do not essentially differ from those of the middle-aged adult population, but we must consider that the body's vitamin stores might get filled up, which may reduce vitamin needs, on the one part, but the altered physiological processes may increase them, on the other. Regarding the case of fat-soluble vitamins, reduced fat absorption, decreased vitamin storage capacity of the liver, reduced dietary intake, partial deficiency of digestive enzymes, and absorption disorders in the intestines may all lead to vitamin deficiencies. Problems may also arise due to multiple vitamin overdose developed either as a consequence of overconsumption of vitamin tablets or because the body's vitamin stores are constantly filled up to maximum capacity. The consumption of several times the daily dose is recommended only in cases of special vitamin deficiencies as there is a high risk of overdose, which could result in serious diseases. It appears that A, D, E, or K vitamin deficiency occurs very rarely in developed countries in the case of a normal diet; great care must be taken, however, in order to meet vitamin A and D and, simultaneously with the latter one, calcium requirements so that to avoid osteoporosis and an increased risk of bone fractures in elderly people.

Although the vitamin A content of the liver is constant in elderly people, the efficiency of releasing vitamin A stored in the liver decreases. In the elderly, the consumption of several times the daily dose recommendations may cause pain in the bone, osteitis, and – due to an increased vitamin D activity induced by vitamin A – hypercalcemia, which decreases bone mineral density and increases predisposition to bone fractures.

Vitamin D requirements of the elderly are heavily dependent on the environment as this vitamin is also produced in the skin upon direct exposure to UV radiation, although this synthesis – as a result of changes in lifestyle – is suppressed in the elderly population. In such cases, the D vitamin content of foodstuffs is of paramount importance, and deficiencies can occur if elderly people do not consume milk and dairy products at all or only in small quantities. Changes in biochemical processes (decreased absorption from the intestines, poor efficiency of hydroxylation in the liver and the kidney) may also cause vitamin deficiencies, whereas use of medication may reduce absorp-

tion as well. Therefore, up to 70 years of age, 10 $\mu$g, whereas beyond this threshold 20 $\mu$g/day of vitamin D appears to be the optimal dose for elderly people, which is advisable to be combined with 200–600 mg of calcium/day to maintain an appropriate condition of the bones. Besides a similar dose of vitamin, many recommend a daily calcium intake of 1,000–2,000 mg, especially in the case of female individuals, where predisposition to osteoporosis is much higher. Hypervitaminosis is very rare for both vitamins, and it can occur only in extreme cases that involve consumption several times the daily vitamin requirements.

In old age, vitamin E and K deficiency almost never occurs in the case of a normal diet. Vitamin E deficiency may occur in cases of malabsorption of fats, pancreatic problems and enteritis, or coeliac disease, consequent upon which a whole range of aetiological diseases (anaemia, coagulation disorders, or neurological symptoms) may develop. We must be careful with regular cases of overdose as they may lead to muscle weakness, exhaustion, nausea, or diarrhoea. As intestinal flora is also a producer of vitamin K, such deficiency would occur only subsequent to a major gastrointestinal surgery. In cases like this, not even multiple vitamin overdose may cause any problem. Daily doses of vitamin K intake are closely linked to the development of healthy bones, as a mild overdose reduces the number of bone fractures.

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# The role of vitamins in the diet of the elderly II. Water-soluble vitamins

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**Abstract.** Following a presentation of humans' water-soluble vitamin requirements, the authors will discuss in detail the role these vitamins play in human organism and outline those major biochemical processes that are negatively affected in the body in case of vitamin deficiency. They point out that in the elderly population of developed countries cases of water-soluble vitamin deficiency are extremely rare and they are due to the lack of dietary vitamin, but mostly to the vitamin being released from its bindings, the difficulty of free vitamin absorption, gastrointestinal problems, medication, and often alcoholism. Among water-soluble vitamins, $B_{12}$ is the only one with a sufficient storage level in the body, capable of preventing deficiency symptoms for a long period of time in

**Keywords and phrases:** B vitamins, vitamin $B_1$, $B_2$, $B_3$, $B_6$, $B_{12}$, thiamine, riboflavin, niacin, folic acid, biotin, pantothenic acid, vitamin C, ascorbic acid, vitamin U, water-soluble vitamins, vitamin requirements, vitamin requirements of the elderly

cases of vitamin-deficient nutrition. Each type of vitamin is dealt with separately in discussing the beneficial outcomes of their overconsumption regarding health, while the authors of the article also present cases with contradictory results. Daily requirements are set forth for every water-soluble vitamin and information is provided on the types of nutrients that help us to the water-soluble vitamins essential for the organism.

# 1 Introduction

Our previous review article reports on humans' protein and energy needs, the role of fat-soluble vitamins in the diet of elderly people. The present paper will treat the water-soluble vitamin needs of elderly people. Beyond generalities, we will discuss the role of each vitamin, the consequences of vitamin deficiency and overuse, and the therapeutic use of vitamins, in each case touching upon the needs of elderly people. *Table 1* shows the RDI values of water-soluble vitamins and the tolerable upper limits (*Thomas*, 2007).

Table 1: RDI values of water-soluble vitamins for the male and female population over 70 years of age and the tolerable upper limits

| Vitamin | Men | Women | Upper limit |
|---|---|---|---|
| Vitamin $B_1$ (Thiamine) (mg) | 1.2 | 1.1 | no data |
| Vitamin $B_2$ (Riboflavin) (mg) | 1.3 | 1.1 | no data |
| Vitamin $B_3$ (Niacin) (mg) | 16 | 14 | 35 |
| Vitamin $B_6$ (Pyridoxine) (mg) | 1.7 | 1.5 | 100 |
| Vitamin $B_{12}$ (Cyanocobalamin) ($\mu$g) | 2.4 | 2.4 | no data |
| Folic acid (mg) | 600 | 600 | 1,000 |
| Biotin (mg) | 30 | 30 | no data |
| Pantothenic acid (mg) | 5 | 5 | no data |
| Vitamin C (Ascorbic acid) (mg) | 90 | 75 | 2,000 |

If the data included in the above table were compared with levels determined for younger people, we could only observe minimal differences, which reminds us of the fact that only certain types of disease as well as cases of indigestion and malabsorption need special attention regarding the vitamin needs of the elderly population.

## 2 Vitamin requirements

Our body does not store up water-soluble vitamins, wherefore it falls to us to take care of the daily vitamin B supply – except for vitamin $B_{12}$ stored in the liver. Due to the facts outlined above, deficiency for vitamin $B_1$ was found in 13–43% and for vitamin $B_6$ in 5–56% of the elderly people (*Hajjar & Pharm*, 2007). Such typical cases of vitamin deficiency diseases as scurvy, beriberi, or pellagra have a very low incidence rate among the senior population. Vitamin deficiencies tend to be symptomatic in cases of insufficient consumption of foods in terms of quantity and quality or when people refuse to consume certain types of food on a whim (*Gordon*, 1993).

## 3 Water-soluble vitamins

### 3.1 Vitamin $B_1$

The first member of the B-group vitamins, vitamin $B_1$ (thiamine, aneurine, thiamine pyrophosphate, TPP), is the coenzyme of enzymes that take part in the citric acid cycle and help the release of chemical energy. The decarboxylation of the pyruvic acid and $\alpha$-ketoglutaric acid into acetyl-coenzyme A and succinyl-coenzyme A are key reactions of this cycle, while both enzymes function with TPP coenzyme. Key enzymes containing TPP can also be found in the pentose phosphate cycle as well as in the synthesis of ATP, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA), listing it as an indispensable vitamin for our organism. Ascertainment of our body's vitamin $B_1$ supply can take place via the measurement of erythrocyte transketolase activity or, more recently, via the direct analysis of erythrocyte TPP content.

Vitamin $B_1$ deficiency causes beriberi disease. Typical symptoms of this avitaminosis are: neuritis; muscle weakness; insomnia; oedema starting in the extremities, and ultimately covering the entire human body; finally, death would set in due to paralyses and cardiac dysfunction. As a consequence of vitamin $B_1$ deficiency, carbohydrate metabolism is disrupted as the intermediary metabolites (pyruvic acid, lactic acid) become concentrated in the tissues and the blood, since the decomposition of the pyruvic acid takes place with the aid of an enzyme containing vitamin $B_1$. Linked to phosphates, vitamin $B_1$ forms thiamine pyrophosphate (TPP) coenzyme – in the case of its deficiency, a dysfunction in the pyruvic acid decarboxylase and transketolase sets in, whose prosthetic group.

Based on its structure, vitamin $B_1$ is also known as thiamine because the molecule contains partly sulphur, partly amino group. Due to its antineuritic properties, it is also called aneurine. This is the most heat sensitive member of the B-group vitamins. It is quickly inactivated in the presence of heavy metal salts, oxidizing agents, and sulphuric acid. The husk and germ of cereal grains is a particularly rich vitamin source, wherefore bread made from wheat flour contains vitamin $B_1$ in very small quantities as opposed to brown bread. The different tissues (kidney, liver, and muscle tissues) contain vitamin $B_1$ in varying quantities depending on the animal species. Yeast disposes of the highest thiamine content. The average diet barely covers the 1.5–2.0 mg daily thiamine requirement of an adult person.

Although a varied diet can cover the vitamin $B_1$ needs of the elderly, experience shows that a great number of elderly people has a poor supply of this vitamin. In an experiment, 46% of the participating elderly individuals had vitamin $B_1$ deficiency as they had been consuming one third less of this vitamin than RDA specifications (*Abraham et al.*, 1979). 5% of retirement home residents and 13–43% of community home residents are poorly supplied with vitamin $B_1$ (*Joshi & Morley*, 2006). The reason for such a low percentage of deficiency in retirement home residents is that individuals at risk are regularly provided with multivitamin supplements, stocking them with sufficient quantities of vitamin $B_1$.

While vitamin $B_1$ supplements several times the RDA value were shown to improve comprehension, another experiment did not yield any improvement in elderly people with Alzheimer's disease. Generally speaking, vitamin $B_1$ supplements have made a valuable contribution to improving the elderly population's quality of life, a fact demonstrated by improved appetite, restful sleep, higher level of physical activity, and reduced level of fatigue. Quantities several times the RDA value are well tolerated by the organism since the excess is eliminated in urine.

In industrialized countries, vitamin deficiency often has its roots in alcoholism, as with the high amount of fluid intake vitamin $B_1$ is expelled from the system via elimination through the kidney (*Suter et al.*, 2000). Vitamin $B_1$ deficiency can also be traced back to kidney problems for haemodialysis or peritoneal dialysis can accelerate the elimination of vitamin $B_1$ from the body (*Hung et al.*, 2001).

### 3.2 Vitamin $B_2$

In the human organism, vitamin $B_2$ (riboflavin, lactoflavin) is the cofactor of flavin mononucleotide (FMN) and of flavin adenine dinucleotide (FAD), wherefore it takes part in the biological oxidation processes, the mitochondrial oxidative phosphorylation, and the synthesis of adenosine triphosphate (ATP). These enzymes are collectively termed as flavoproteins. Entering into reaction with the substrate, it reversibly binds two hydrogen atoms on the two nitrogen atoms, during which flavin is reduced to leucoflavin. It plays a vital role in the production of reduced glutathione, an excellent reduction agent of the organism. It also takes part in the detoxification processes in the liver, through which the body neutralizes toxic substances and various drugs.

Vitamin $B_2$ deficiency causes inflammatory symptoms and cracking to appear on the mucous membrane of the mouth and tongue, while general fatigue and visual disturbances also manifest themselves. This deficiency is present in many places all over the world in people who struggle with persistent diarrhoea, have liver problems, or are alcoholics. There are rare cases of specifically vitamin $B_2$ deficiency; much rather, the combined deficiency of several members of the B-group vitamins triggers clinical symptoms.

There are few studies on the elderly people's vitamin $B_2$ requirements. Broadly speaking, the oxidative damage of the lens proteins can be prevented by increasing the nutrients' antioxidant components – in this context, the risk of cataract development was shown to be inversely proportional to riboflavin intake (*Cumming et al.*, 2000). It has also been established that in the prevention of cataract development not only riboflavin but all compounds with antioxidant properties introduced into the body are efficient.

Vitamin $B_2$ is not sensitive to heat, but it easily decomposes upon exposure to light, via a photochemical reaction entailing the separation of the side chain and resulting in the production of the biologically inactive alloxasine derivatives. Riboflavin is widely spread in plant and animal tissues as well as in various foodstuffs. Vitamin $B_2$ content is especially high in liver, kidney, fish, egg, milk, and different vegetables. Milk is particularly rich in this vitamin, which is why it is also called lactoflavin. Humans' daily vitamin $B_2$ requirements are 1.5–2.0 mg.

### 3.3 Vitamin $B_3$

The deficiency disease of vitamin $B_3$ (nicotinic acid amide, niacin, vitamin PP) is pellagra, which first of all occurs in people who mostly consume corn-based foodstuffs. This disease starts off with symptoms of general fatigue, then a

dysfunction of the gastrointestinal tract develops, inflammatory cracking appears on the mucous membrane of the mouth and tongue, and the skin surface becomes rough, inflammatory, and exfoliative. The above listed symptoms will subside if nicotinic acid amide is administered, but tryptophan, thiamine, and riboflavin intake are also necessary to achieve a complete recovery.

Regarding its chemical structure, vitamin PP is nicotinic acid, also called niacin, whereas in natural substances we can find nicotinic acid amide, i.e. niacinamide. The living organism can easily amidate nicotinic acid. Niacin is widely spread in nature for it can be found in its coenzyme form in all living cells. High amounts of it can be found in the husk of cereal grains, in yeast, liver, kidney, the meat of animals and fish, milk, egg, and vegetables. Some of the niacin can be found in protein-bound form, and therefore this part of the cereals' niacin content – since a release cannot take place but via alkaline hydrolysis – will not be bioavailable by way of nutrient intake (*Gregory*, 1998). Corn has very small amounts of nicotinic acid amide and tryptophan, leading to frequent cases of pellagra in those living on a corn-based diet.

Nicotinic acid amide is integrated into the dinucleotide coenzyme (NAD, NADP) of the pyridine enzymes. As one of the double bonds is disrupted, the pyridine ring takes up hydrogen, this way becoming part of the enzymatic action. Besides this, it has a vital role in producing the hydrochloric acid of the gastric juice and lowering cholesterol levels in the blood, while also having vasodilatory effects. In the presence of vitamins $B_1$, $B_2$, and $B_6$, the liver is able to synthesise it from tryptophan, but with a very low conversion efficiency as for the biosynthesis of 1 mg of niacin 60 mg of tryptophan is required.

The daily nicotinamide requirement of an adult person is 10–20 mg. Its RDA value for vitamin $B_3$ is 14 mg/day for women over 50 years of age and 16 mg/day for men of the same age-group. Gastric ulcer, enteritis, or alcoholism may significantly increase these values. For treating cardiovascular symptoms and, formerly, reducing the cholesterol level in the serum, doses of 500–3,000 mg/day were efficiently applied. It has now become clear that an increased quantity of vitamin $B_3$ increases the high-density lipoprotein level (HDL) in the serum, reduces triglyceride and lipoprotein(a) levels, and – to a lesser degree – reduces the level of low-density lipoprotein (LDL) as well as the total amount of cholesterol (*Brown et al.*, 2001). When administered alone, vitamin $B_3$ reduced the rate of cardiovascular diseases (*Canner et al.*, 1986). Medication aimed at reducing LDL cholesterol level is more efficient if administered along vitamin $B_3$.

### 3.4 Vitamin $B_6$

Hypervitaminosis of vitamin $B_6$ (pyridoxine) causes disturbances in protein metabolism. Its deficiency induces symptoms reminding of pellagra: the mouth and the eyes turn red, inflammation appears, the skin becomes chapped and exfoliative, and hair falls out. The term pyridoxine comprises three compounds with similar vitamin effects: pyridoxol, pyridoxal, and pyridoxamine – all of them being substituted pyridine derivatives. Pyridoxol can be found in foods of plant origin, whereas pyridoxal and pyridoxamine in foods of animal origin as phosphate ester. Phosphoric acid is linked to the primary alcoholic hydroxyl group at position 5, and so vitamin $B_6$ exerts its physiological effect in the form of pyridoxal-5-phosphate (PLP).

It plays a biological role in the intermediary metabolism of the amino acids, where its phosphoric acid esters are the coenzymes of various enzymes (aminotransferases, amino acid decarboxylases, desulphurylases, etc.). These enzymes take part in the synthesis of amino acids and neurotransmitters (serotonin, norepinephrine, $\gamma$-amino butyric acid). PLP is required for the synthesis of nicotinic acid from tryptophan and it is the coenzyme of glycogen phosphorylase, which releases glucose from glycogen; it takes part in glycogenesis as well, during which sugar is created from amino acids.

Meat, fish, liver, egg yolk, whole grains, vitamin-supplemented cereals, leafy vegetables, leguminous plants are all sources of vitamin $B_6$, but intestinal bacteria also produce it. Daily requirements of an adult person amount to 2–3 mg, but as we get older these needs may be higher. Vitamin $B_6$ of plant origin can be found in glycosidic linkage, whose availability is limited. It is absorbed in the first segment of the small intestine via passive transport, which is aided by the acidic environment. Since our body cannot store it, a regular intake is necessary on a daily basis. Vitamin $B_6$ deficiency occurs when consuming vitamin-deficient foods, in cases of cirrhosis, inadequate dialysis, or with alcoholics. Certain medications can also trigger vitamin deficiency, in which case dietary intake must be supplemented with vitamin $B_6$.

Along with other digestive problems, vitamin $B_6$ deficiency often occurs in elderly people (*Johnson*, 1995), increasing the homocysteine level in the serum, which is generally acknowledged to increase the risk of cardiovascular diseases (*Eikelboom et al.*, 1999). Several researchers reported vitamin $B_6$ intake in quantities much larger than the RDA value to protect against cardiovascular diseases and high cholesterol level, while also having beneficial effects for diabetic patients. The co-administration of vitamin $B_6$ and folic acid has probably reduced the incidence of coronary artery disease (*Folsom et al.*, 1998).

In the case of vitamin $B_6$ deficiency, the immune system can also be compromised as the affected person may have to face a considerably low lymphocyte count and likewise a low interleukin production. It has been established that the normal functioning of the immune system can be restored by applying several times the recommended RDA value (*Meydani et al.*, 1991). A close negative correlation was found in the elderly between cognitive abilities, high homocysteine as well as low vitamin $B_6$ levels. With doses of vitamin $B_6$ (20 mg/day) exceeding RDA values (1.7 mg/day), a definite improvement was observed regarding the memory-related abilities of elderly people aged 70–79 years (*Bryan et al.*, 2002).

The excess of vitamin $B_6$ is oxidized into pyridoxine acid, and is eliminated from the body.

### 3.5 Pantothenic acid

Pantothenic acid deficiency causes fatigue, agitation, muscle cramps, and indigestion. Its avitaminosis inhibits growth, reproduction, causes pellagra-like mutations, subcutaneous bleeding, and neural dysfunctions. Pantothenic acid is in fact the peptide of the pantoic acid, formed by beta-alanine. It is an acidic water-soluble compound, which under neutral conditions is resistant to light and the oxygen in the air. It is inactivated upon exposure to strong acidic or alkaline substances. Natural occurrences are also observed in the case of pantothenol, which is the alcohol analogue of pantothenic acid, and pantotheine, which is the decarboxylation product of pantothenol-cysteine resulting from the linkage of pantothenic acid and cysteine. Both of the compounds act as pantothenic acid.

Pantothenic acid performs its biological effect in metabolism as a constituent part of coenzyme A, acting as an indispensable contributor to the utilization of energy-providing nutrients and also controlling the interconversion of fats and carbohydrates. Coenzyme A is in fact created through the union of pantetheine and ADP. We have knowledge of more than 70 enzymatic reactions in which it takes an active part. The best known of them are the transfer of the acyl group during the decomposition of carbohydrates, fatty acids, and amino acids and the participation in the biosynthesis of fatty acids as well as porphyrin and steroid compounds.

### 3.6 Folic acid

Folic acid (folate, pteroylglutamic acid) occurs in high quantities in the leaves of green plants. Its deficiency in the human organism causes anaemia since

folic acid along with vitamin $B_{12}$ regulates the formation of red and white blood cells and of platelets. Additionally, it plays a role in developing the mucous membrane of the gastrointestinal tract. The term folic acid is used for a class of compounds whose parent compound is pteroic acid and the 1, 3, or 7 glutamic acid molecules linked to it. Pteroic acid is the derivative of para-aminobenzoic acid and pteridine. It is the first glutamic acid linked through amide bond, while the rest are linked through $\gamma$-peptide bond – they are termed pteroylglutamic acid, pteroyltriglutamic acid, and pteroylheptaglutamic acid. Induced by ascorbic acid and NADPH, pteroylglutamic acid forms 5,6,7,8-tetrahydrofolic acid, which will biologically transform into active folinic acid.

Folinic acid takes part in building up the coenzyme of synthetases, transferases, and isomerases. Enzymes play an active role in the transfer of one-carbon units; as carbon donors, they are essential in the synthesis of amino acids and nucleic acids as well. One of the most important processes is homocysteine remethylation to methionine, which requires folic acid and vitamin $B_{12}$. In their absence, the homocysteine level in the serum increases, just as the risk of developing cardiovascular diseases.

Folic acid can be absorbed from the small intestine both via passive and active transport. From the liver, it re-enters the bloodstream, while the excess is eliminated from the body via the kidney, wherefore our organism cannot store larger quantities of folic acid. Following the reduced intake of folic acid, its deficiency will manifest within a few days although clinical symptoms take a lot more time to appear. The incidence rate of folic acid deficiency in the elderly population is 2–34% (*Joshi & Morley*, 2006). Besides a vitamin-deficient diet and malabsorption, excessive alcohol consumption, smoking, atrophic gastritis, enteritis, and certain medications can also contribute to a state of avitaminosis (*Kishi et al.*, 1997). Since folic acid deficiency in the elderly can be linked to the development of diseases such as various types of cancer, arteriosclerosis, or senile dementia, the relevant daily RDA value was increased to 400 $\mu$g. A daily intake of 5 mg folic acid was shown to reduce the development of colon cancer and other intestinal tumours (*Kim et al.*, 2001; *Su & Arab*, 2001). Two servings of alcohol per day coupled with folic acid deficiency also increased the risk of developing colon cancer. An increased folic acid supplement reduced the incidence of developing breast cancer in alcoholic women (*Rohan et al.*, 2000).

Recently, there have been several studies on the connection between folic acid intake and the deterioration of cognitive abilities in elderly people. Researchers pointed out that folic acid deficiency in people aged over 65 years

is accompanied by short-term memory impairment, which is a form of senile dementia (*Ebly et al.*, 1998). Upon administering folic acid supplement, a reduced risk of developing ischemic stroke can be observed (*He et al.*, 2004).

People's normal diet usually contains sufficient amounts of folic acid. The food sources richest in folic acid are liver, kidney, meat, various mushrooms, asparagus, Brussels sprouts, citrus fruits, and leafy vegetables. Folates are highly sensitive to oxidation and heat; therefore, in the process of preparing food, 50–95% of the basic material decomposes, but folic acid – the biologically active form of the vitamin – is essentially more stable (*Johnson et al.*, 2002). Intestinal flora also contributes to the 0.4 mg of folic acid necessary for the normal functioning of the human organism.

There are no known adverse effects of excessive folic acid intake, but its consumption in high quantities over a longer period of time can lead to vitamin $B_{12}$ deficiency and may even cause irreversible neurological damage, which is why a daily folic acid intake exceeding 1,000 $\mu$g is not advised (*Higdon*, 2002).

### 3.7 Biotin

The avitaminosis of biotin (vitamin H) causes loss of appetite, dermatitis, hair loss, and greasy skin surface. It is an important growth factor for saccharomyces cerevisiae as well. The biotin molecule is made up of a sulphurous annular portion and valeric acid side chain. The structure made up of two 5-membered heterocycles is formed through the linkage of carbamide and thiophene ring. Biotinal, the aldehyde version is also biologically active and can be oxidized to biotin. We can also find in nature its acid amide formed with lysine, the likewise active biocytin.

Biotin is not heat sensitive, but it decomposes upon exposure to strong acidic and alkaline substances or oxidizing agents, while it is slowly inactivated when exposed to light. Avidin glycoprotein found in raw egg white forms such a complex with biotin that can resist enzymatic breakdown, and thus the avidin-biotin complex is eliminated from the body through the digestive tract. Biotin can be found in plants in free form, while in animal tissues, microorganisms, and dairy products its protein-bound form can be observed. Humans' daily requirement amounts to 100–300 $\mu$g. In terms of human nutrition, the most important sources are represented by liver, kidney, milk, egg yolk, soy, vegetables, nut, and yeast, while intestinal flora can also synthesise biotin. Specialized literature does not mention any data on elderly people's biotin needs.

Its biological function is carried out in the role of the enzymatic prosthetic group, linked to the lysine part in the enzyme protein peptide chain. Biotin-containing enzymes take part, first of all, in the decarboxylation, deamination, carboxylation, and synthetisation processes essential in carbohydrate and lipid metabolism.

### 3.8 Vitamin $B_{12}$

Human organism needs vitamin $B_{12}$ (cobalamin) for a normal growth, a healthy nervous system, and haematopoiesis. Its central structure is the corrin ring, similar to the porphyrin shell and forming a girdle around the cobalt atom. Via complex bonding, 5,6-dimethylbenzimidazole and a cyanide or a hydroxyl or a nitrite radical are linked to the cobalt atom. All three forms have vitamin effects, since following absorption these radicals are equally replaced by 5-deoxyadenosine, and cobalamin is incorporated into the enzyme in this form (adenosylcobalamin) (*Higdon*, 2003).

It takes part as a coenzyme in several important processes (thiamine synthesis, reduction of one-carbon units, propionic acid metabolism, etc.). In several biochemical processes, it is present together with folic acid. It is the cofactor of methionine synthase and L-methylmalonyl-CoA mutase. The former enzyme converts homocysteine into methionine, which is required for the synthesis of S-adenosylmethionine, and it takes part as a methyl group donor in a number of biochemical reactions where the presence of a methyl group is necessary, such as RNA and DNA methylation. It controls the synthesis of L-methylmalonyl-CoA mutase and succinyl-CoA from methylmalonyl-CoA.

A lesser degree of vitamin $B_{12}$ deficiency causes neurological disorders, whereas high levels of vitamin $B_{12}$ deficiency lead to pernicious anaemia, whose typical symptoms are reduced red blood cell count and the unnatural swelling these cells; additionally, loss of appetite, weakness, and indigestion can also occur. The absorption of vitamin $B_{12}$ requires mucoprotein produced in the gastric wall and with a molecular weight of 60,000 daltons, which can release vitamin $B_{12}$, introduced into the body through dietary intake, from protein complexes, and helps the linking of appropriate receptors in the small intestine.

Vitamin $B_{12}$ is exclusively manufactured by microorganisms – it cannot be found in plants and yeast, while the intestinal microorganisms satisfy the needs of herbivorous animals. The consumption of foods of animal origin with high protein content helps humans to the necessary vitamin $B_{12}$, regarding which the daily requirement is 3–4 $\mu$g. In the case of elderly people, the RDA value for vitamin $B_{12}$ is 2–4 $\mu$g, about half of which can be covered by consuming

half a litre of milk or a corresponding dairy product. Milk is an extremely important source of vitamin $B_{12}$ for vegetarians too as without it they cannot obtain this essential substance.

Gastric acid and enzymes are required in order for vitamin $B_{12}$ to be released from its bonds and become available via absorption. Pancreatic proteases break down proteins, and as a consequence the released vitamin $B_{12}$ enters the bloodstream, and through a transport mechanism gets into the liver, where it is stored up. Vitamin $B_{12}$ deficiency in the elderly – except for vegetarians – can be associated with low levels of vitamin ($B_{12}$) absorption. Since the human body is able to store it in the liver, where a sort of a rescue reaction is also present, it has a long half-life in this organ, and therefore deficiency diseases do not develop until a few years' time. It is estimated that 4–43% of the elderly population suffers from vitamin deficiency.

As gastric acid and digestive enzymes are needed to release vitamin $B_{12}$ from protein, gastric acid deficiency leads to vitamin deficiency. Gastritis is very frequent in elderly people (30%): it reduces absorption, which leads to vitamin deficiency. High bacteria count in the small intestine due to gastric acid deficiency can also lead to vitamin deficiency, as bacteria use up vitamin $B_{12}$ for reproduction. Vitamin $B_{12}$ deficiency in the elderly population can occur consequent upon pancreatic enzyme production activities or following gastrointestinal surgery (*Joshi & Morley*, 2006). This may cause functional disturbances of the central nervous system, forgetfulness, and depression in the elderly.

As an established procedure, cyanocobalamin intramuscular injection is prescribed for cases of vitamin malabsorption. As the body is capable of storing it, a dose of 1,000 $\mu$g administered every month or every few months may be the solution to the problem (*Hajjar et al.*, 2000), though not a preferred procedure anymore. For explicit anaemia, it is recommended that the patient take 1,000 $\mu$g of vitamin $B_{12}$ instead of the usual daily doses of 100–500 $\mu$g. Similar to vitamin $B_6$ deficiency, low levels of vitamin $B_{12}$ also increase the homocysteine level in the serum, enhancing susceptibility to cardiovascular diseases. Like with folic acid, 500 $\mu$g/day of vitamin $B_{12}$ supplement could also reduce the homocysteine level significantly (*Stabler et al.*, 1997).

Decreasing levels of vitamin $B_{12}$ in the serum were accompanied by increasing homocysteine levels, which could be successfully compensated for by an amount of vitamin $B_{12}$ 3–5 times the RDA value. Vitamin $B_{12}$ deficiency was associated with senile Alzheimer's disease and showed a strong correlation with depression symptoms over 65 years of age.

### 3.9 Vitamin $B_{15}$

Vitamin $B_{15}$ (pangamic acid) is an essential methylating agent of the living organism. Its physiological importance is due to the role it plays in assisting tissue oxygen metabolism, while also having detoxifying and lipotropic effects. Those substances have lipotropic properties that prevent certain organs (the liver) from lipomatosis. Its chemical composition: it is an ester of D-gluconic acid and dimethylglycine. In our foodstuffs, it can be found in large quantities in liver, yeast, and treacle. There are no data recorded in literature on elderly people's pangamic acid requirements.

### 3.10 Vitamin U

Vitamin U (S-methylmethionine) cures gastric ulcer as well as inhibits its formation, lowers lipid and cholesterol levels in the serum, and its lipotropic effect is similar to that of pangamic acid. In terms of chemical composition, it is the sulphur-methylated, L-configuration, basic, reactive sulphonium derivative of methionine. In higher plants, it is formed from S-adenosylmethionine. In the human organism, it takes part in the synthesis of choline and creatine. It is an essential methylating agent, a substitute for methionine. The human body cannot synthesise a methyl group, which is why it needs methyl donors to break down fats. Vitamin U can be found in cabbage, lettuce, tomato, spring onion, radish, leaf parsley, asparagus, and fruits. As a result of cooking, it breaks down into dimethyl sulphide and homoserine; therefore, raw vegetables and their expressed juices are in particular rich sources of vitamin U. There are no data recorded in literature on elderly people's vitamin U requirements.

### 3.11 Vitamin C

Scurvy developed as a consequence of vitamin C (ascorbic acid) deficiency was a disease feared by everyone in the Middle Ages, but survey results showed that scurvy is actually the outcome of the combined deficiency of vitamin C and certain bioflavonoids. Its characteristic signs are general fatigue, shortness of breath, cardiac dysfunction, pain in the muscles and bones, increased gingival bleeding, and then petechial haemorrhaging on the lower extremities caused by the vulnerability of the capillary vessels and increased capillary permeability. The bones become fragile, the joints swell up, the teeth become loose and fall out, wounds heal very slowly, and finally death sets in. Nowadays, vitamin C deficiency would only cause the development of the so-called spring fever, consequent upon which the immunity of the body decreases and at the same time susceptibility to colds increases.

Scurvy can develop only as an outcome of vitamin C intake not exceeding 10 mg/day throughout a period of at least three months. This disease usually goes along with poor-quality dentine, which will cause the teeth to fall out. In the case of vitamin C deficiency, iron-deficiency anaemia can also occur.

The abovementioned symptoms and diseases come as a corollary to the fact that vitamin C is necessary for the synthesis and integrity of the collagen, a crucially essential contributor to the development of blood vessels, tendons, and ligaments as well as to wound healing. Vitamin C deficiency results in abnormal collagen cross-linking, reducing its tensile strength (*Joshi* & *Morley*, 2006). Altered collagen structure may lead to abnormal bone formation. It also plays an important role in norepinephrine and carnitine synthesis. Carnitine helps fatty acids enter the mitochondria, this way assisting energy release. Vitamin C is highly efficient in fighting off free radicals attacking the body, which are formed in our everyday life as a consequence of contaminants, toxic substances or as a result of smoking. Vitamin C protects our DNA, proteins, unsaturated fatty acids, and carbohydrates from oxidation. It helps the duodenal absorption of iron and other microelements, the reduction of methemoglobin to haemoglobin, and it reduces the nitrates, this way inhibiting the development of mutagenic nitrosamines, and thus preventing cancer.

The average daily vitamin C requirement for an adult person, depending on the (type and amount of) work carried out, is cc. 80–100 mg. Although the excess of vitamin C is eliminated in urine, its excessive consumption is detrimental to health (due to the formation of renal calculi). The experiments of Svirberly and Szent-Györgyi (1932) demonstrated that administering ascorbic acid on its own would not put an end to the increased permeability of blood vessels, haemophilia. In order for this to happen, we need the help of vitamin P, first manufactured in 1936 for the regulation of permeability. Bioflavonoid glycosides have vitamin P effects, and among them rutin proved to be biologically the most active.

Rutin is a glycoside whose sugar moiety is rutinose and its aglycone is a three-ringed flavonol compound. Rutinose is an oligosaccharide formed from D-glucose and L-rhamnose via a $\beta$-configuration binding. (Bioflavonoids are not included in the category of vitamins anymore.)

Given that it is a water-soluble vitamin, excessive vitamin C intake can have very few adverse effects. However, a daily vitamin C intake of over 2,000 mg can lead to gastrointestinal symptoms such as nausea, abdominal cramps, and diarrhoea. Abnormal vitamin $B_{12}$ absorption and elevated blood oestrogen levels may also occur in these cases (*Miller* & *Hays*, 1982). It is considered by many a medicine against common cold although experiments have shown

that its consumption in daily doses of 2,000 mg has no appreciable effect on mild cases of cold, but it has reduced the duration of common cold diseases in adult individuals by 8%.

Some claim that high doses of vitamin C intake help prevent the onset of cardiovascular symptoms as well as cancer in older people, but these allegations have not yet been confirmed. However, it appears to be certain that it works as an efficient factor in preventing the opalescence of the lens among members of the senior population (*Mares-Perlman et al.*, 2000). Since the beneficial effects of vitamin C megadosage are not evident, the administration of such large doses is not practical. Based on current knowledge, a daily vitamin C intake of 100–140 mg seems to be a sufficient amount for this vitamin to access all essential points in our body and prevent any harmful processes caused by its deficiency. By consuming foods of natural origin rich in vitamin C (five servings of fruits and vegetables per day), we may even achieve a daily vitamin C intake of 200 mg, which can significantly reduce the risk of cancer in elderly people.

Despite there is no biochemical system in our body that could store vitamin C, adrenal glands, the pituitary gland, the thymus, and the retina may contain as much as 50 times the amount found in the serum. The brain, the placenta, the testicles, the thyroid gland, the pancreas, and the kidneys all contain 5–25 times more vitamin C than the serum (*Jakob*, 1999). Generally speaking, it can be concluded that women's serum contains 20% more vitamin C compared to men and that non-smokers are better supplied in this regard than their smoking peers. Consequently, the RDA value for men is 90 mg/day, for women 75 mg/day, while for smokers is 35 mg/day more than for non-smokers (*Johnson et al.*, 2002).

Vitamin C is the L-configuration lactone of the 2-keto-gulonic acid, the oxidation product of glucose. The 2-keto-gulonic acid also has vitamin effects, but the biological effect of D-ascorbic acid is insignificant. Dienyl group is typically found with ascorbic acid and it can be oxidized to diketo group, wherefore ascorbic acid is a strong reducing agent; as a characteristic feature, it is reversibly oxidized to dehydroascorbic acid together with which it forms a redox system. Both forms have vitamin effects, but ascorbic acid is considered to be the more valuable product. Ascorbic acid is converted into diketogulonic acid via oxidation, which, in its turn, breaks down into oxalic acid and L-threonic acid, and these are irreversible transformations. Ascorbic acid is oxidized not only upon exposure to the oxygen in the air or certain chemicals but also by certain enzymatic reactions. Vitamin inactivation is catalysed by heating, light, and trace metals. In the presence of amino acids, ascorbic

acid, dehydroascorbic acid, and its various decomposition products may be converted via Maillard reactions into brown-coloured products. Ascorbic acid typically has a sour taste, it is a crystalline material easily soluble in water, the pH value of its 2% aqueous solution is 2.8, its acidic solutions are stable, but in the presence of air it decomposes above pH 4. The hydrogen of its hydroxyl group on the second carbon atom ($pK_1 = 4.2$) dissociates easily, while the one on the third carbon atom dissociates hardly ($pK_2 = 11.6$). Its primary alcoholic hydroxyl group can be esterified with fatty acid; its ester formed with palmitic acid, ascorbyl palmitate, has antioxidant effect in fats. The stereoisomers of ascorbic acid (D-ascorbic acid, D-isoascorbic acid, D-araboascorbic acid) are antioxidants without any vitamin effects.

The artificial chemical synthesis of vitamin C starts out from D-glucose, followed by the conversion of D-sorbitol into L-ascorbic acid. Green pepper, tomato, cabbage, blackcurrant, fresh and dried rose hip, potato, parsley, and the various tropical fruits particularly abound in vitamin C. Among animal tissues, only offal contains considerable quantities of vitamin C, while the vitamin C content of cow milk is again very low. The vitamin C content of foodstuffs depends on the type of food, the season, the food production technology, and especially the type of heat treatment; therefore, heat-processed vegetables and vegetable soups are not exactly proper sources of vitamin C (*Johnson et al.*, 2002). During storage, the vitamin C content of leafy vegetables can drop by 50–80% compared to their fresh counterparts.

Vitamin C is absorbed from the small intestine via energy-intensive active transport. Its absorption and bioavailability are inversely proportional to the amount of intake. When administered in small doses, vitamin C has a higher bioavailability, while absorption is also influenced by the composition of the food consumed. If we increase the dosage, absorption will decrease although the amount absorbed will increase due to the larger doses (*Levine et al.*, 1999). The half-life of vitamin C in the human organism is very short: 30–60 minutes. The low vitamin content of the serum is derived either from reduced absorption or rapid elimination. Absorption may be affected by pharmaceuticals such as aspirin, which was shown to reduce the vitamin C content of white blood cells by 50%, a fact that can be accounted for by the elimination via the kidney.

The biological effect of ascorbic acid is closely related to its oxidation-reduction potential. In the digestive tract, it stimulates iron and calcium absorption, and it takes part in cellular biochemical processes by maintaining the reduced condition and acting as hydrogen donor. It contributes to connective tissue collagen formation, the synthesis of adrenal hormones, serotonin tissue hormone production, and the oxidative degradation of tyrosine.

# 4 Conclusions

Our body cannot store up water-soluble vitamins, with the sole exception of vitamin $B_{12}$, and therefore various doses of vitamin intake are necessary on a daily basis or, in some cases, several times a week. Water-soluble vitamin intake is also important for the elderly population because many of them are highly efficient against free radicals and due to their antioxidant properties they slow down the ageing process. Some specialists urge the elderly to consume vitamins many times the daily requirements, which is, however, reasonable only during certain diseases; otherwise, the suggested quantities would verge upon hypervitaminosis.

Vitamin B deficiency rarely occurs among the elderly in developed countries as, for the most part, foods can cover vitamin needs and elderly people take vitamin tablets on a daily basis, which contain these in optimal quantities. Vitamin deficiency can be caused by alcoholism, on the one hand, since the high volume of fluid leaving the body eliminates large amounts of vitamin via the kidney, and by different kidney problems, on the other hand, these being related to the pathological nature of dialysis.

Almost all members of the B-group vitamins were established to have beneficial effects on the body in cases of slight overdose or, occasionally, even multiple overdose. Vitamin $B_1$ supplement was shown to improve cognitive abilities in elderly people, increase their activity level and appetite, relax their sleeping, and altogether contribute to improving the quality of life. The human organism proved to have a good toleration for amounts several times the RDA specifications. Vitamin $B_2$ supplement can help prevent the oxidative damage of lens proteins and the development of cataract.

Niacin requirements can significantly increase in old age in cases of gastric ulcer, enteritis, or alcoholism. Supplement intake of more than ten times the necessary amount will increase the level of high-density lipoprotein in the serum, whereas it will decrease the concentrations of low-density lipoprotein, triglyceride, and total cholesterol. When administered alone, it reduced the incidence of cardiovascular diseases and the efficiency of medications lowering LDL cholesterol level.

Vitamin $B_6$ deficiency may exhibit a higher frequency with elderly people and, accompanied by indigestion, increases the homocysteine level in the serum, which entails an increased cardiovascular risk. Its consumption in quantities well above the daily requirement lowers the cholesterol level in the serum, has positive effects in diabetics, and protects against cardiovascular diseases. Its overdose can help restore the normal functioning of a compromised

immune system and has shown a marked improvement in terms of memory in elderly people. If administered together with folic acid, it improves coronary artery condition.

Pantothenic acid deficiency in the elderly can occur rarely, mostly in cases of malnutrition. The primary causes of folic acid deficiency are malnutrition, malabsorption, alcoholism, atrophic gastritis, enteritis, and certain medications. Elderly people's daily needs can be covered with doses of 0.4 mg, but increasing this amount to 5 mg can reduce the risk of colon cancer, other malignant tumours, arteriosclerosis, and senile dementia. Folic acid supplement intake reduces ischemic stroke and the risk of short-term memory impairment. Its excessive consumption is not recommended since that would lead to vitamin $B_{12}$ deficiency and irreversible neurological damage.

Biotin deficiency does not usually occur in old age as foods contain it in sufficient amounts and intestinal flora synthesises it too. In the case of a mixed diet containing foods of animal origin in sufficient amounts, vitamin $B_{12}$ deficiency does not occur as a rule. What causes deficiency in elderly people is that normally gastric acid and enzymes help the vitamin to release from its bindings prior to absorption, which mechanism, however, does not work properly in old age. Entering the liver, it is stored up; consequently, vitamin deficiency is only to be reckoned with if the gastrointestinal absorption of the vitamin decreases. With vitamin storage level at its maximum, deficiency diseases may not even develop but in several years' time. In cases of vitamin $B_{12}$ deficiency, functional disturbances of the central nervous system, depression, and forgetfulness may occur, while due to the increased serum homocysteine level the risk of developing cardiovascular diseases may also become higher. Vitamin $B_{12}$ and U deficiency occurs in extremely rare cases involving elderly people.

As for vitamin C efficiency, the beneficial or adverse effects of its overdose, the results are highly contradictory. Some claim that high doses (1,000 mg or more per day) of vitamin C intake help prevent the onset of cardiovascular symptoms and cancer in elderly people, whereas others have not found such beneficial effects. It appears to be certain that several times the required dosage is efficient in preventing the opalescence of the lens, but a daily vitamin C intake exceeding 100–140 mg is not recommended by most. Consumption above 2,000 mg/day may even trigger gastrointestinal symptoms.

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