# Agronomy Research

Established in 2003 by the Faculty of Agronomy, Estonian Agricultural University

## Aims and Scope:

*Agronomy Research* is a peer-reviewed international Journal intended for publication of broad-spectrum original articles, reviews and short communications on actual problems of modern biosystems engineering incl. crop and animal science, genetics, economics, farm- and production engineering, environmental aspects, agro-ecology, renewable energy and bioenergy etc. in the temperate regions of the world.

## Copyright:



## *Agronomy Research* online:

*Agronomy Research* is available online at: http://agronomy.emu.ee/

## Acknowledgement to Referees:

The Editors of *Agronomy Research* would like to thank the many scientists who gave so generously of their time and expertise to referee papers submitted to the Journal.

## Abstracted and indexed:

SCOPUS, EBSCO, CABI Full Paper and Thompson Scientific database: (Zoological Records, Biological Abstracts and Biosis Previews, AGRIS, ISPI, CAB Abstracts, AGRICOLA (NAL; USA), VINITI, INIST-PASCAL.)

## Subscription information:

Institute of Technology, EULS
St. Kreutzwaldi 56, 51014 Tartu, ESTONIA
E-mail: timo.kikas@emu.ee

### Journal Policies:

Estonian University of Life Sciences, Estonian Research Institute of Agriculture, Latvia University of Agriculture, Aleksandras Stulginskis University, Lithuanian Institute of Agriculture and Lithuanian Institute of Horticulture and Editors of *Agronomy Research* assume no responsibility for views, statements and opinions expressed by contributors. Any reference to a pesticide, fertiliser, cultivar or other commercial or proprietary product does not constitute a recommendation or an endorsement of its use by the author(s), their institution or any person connected with preparation, publication or distribution of this Journal.

**ISSN 1406-894X**

# CONTENTS

**A. Aboltins and J. Palabinskis**
Studies of vegetable drying process in infrared film dryer.....1259

**E. Akk, T.E. Søndergaard, J.L. Sørensen, H. Giese, M.L. Kütt, L. Edesi, H. Lõiveke and E. Lauringson**
The effects of nitrogen rates and intercropping on the occurrence of *Fusarium* spp. on barley kernels.....1267

**N. Dubrovskaya, O. Savkina, L. Kuznetsova and O. Parakhina**
Development of gluten-free bread with unconventional raw ingredients of high nutritional value and antimicrobial activity.....1276

**M. Eliášová and L. Paznocht**
Total phenolic content and antioxidant activity of tritordeum wheat and barley .....1287

**R. Fatkullin, N. Popova, I. Kalinina and V. Botvinnikova**
Application of ultrasonic waves for the improvement of particle dispersion in drinks .....1295

**A. Kirse, R. Galoburda, S. Muizniece-Brasava, D. Karklina and L. Skudra**
Improvement of microbiological safety and shelf-life of pulse spreads through *sous vide* and high pressure processing .....1304

**L. Klavins, J. Kviesis and M. Klavins**
Comparison of methods of extraction of phenolic compounds from American cranberry (*Vaccinium macrocarpon* L.) press residues .....1316

**M. Kremenevskaya, O. Sosnina, A. Semenova, I. Udina and A. Glazova**
Meat industry by-products for berry crops and food production quality improvement .....1330

**L. Kuznetsova and O. Savkina**
A study of factors which influence mould spoilage in flat (sourdough) bread.....1348

**N. Moiseev, E. Suchkova, N. Iakovchenko**
Possibility of using reconstituted milk in manufacture of cheese with cheddaring and cheese curd stretching.....1358

**V. Nazarova and O. Zhdanova**
Development of a rapid method for determination of gluten content in wheat flour 1369

**N. Naumenko,* A. Paymulina, A. Ruskina and V. Khudyakov**
Effects of various raw ingredients on bread quality .....1375

**L. Nilova, N. Naumenko and I. Kalinina**
A study of the forms of bound water in bread and bakery products using differential thermal analysis .....1386

**L. Nilova, T. Pilipenko and S. Malyutenkova**
An investigation into the effects of bioactive substances from vegetable oils on the antioxidant properties of bakery products .....1399

**S. Petrovska1, D. Jonkus1, J. Zagorska and I. Ciprovica**
The influence of k-casein genotype on the coagulation properties of milk collected from the local Latvian cow breeds .....1411

**L. Tsirulnichenko, I.Potoroko, O. Krasulya and I. Gudina**
Increasing the level of hydration of biopolymers in meat processing systems based on the use of acoustically activated brines .....1419

**P. Vaculik, A. Smejtkova, M. Prikryl, O. Drabek, Z. Votruba and L. Lexa**
Selected wastewater parameters from the vegetable washing process .....1426

# FOOD SCIENCE and TECHNOLOGY

*Agronomy Research* **15**(S2), 1259–1266, 2017

# Studies of vegetable drying process in infrared film dryer

A. Aboltins and J. Palabinskis*

Latvia University of Agriculture, Institute of Agricultural Machinery, Cakstes blvd.5, Jelgava, LV – 3001, Latvia
*Correspondence: janis.palabinskis@llu.lv

**Abstract.** The research work analyzes the two fresh vegetable (carrot and garlic slices) drying process in the infrared film dryer. The energy of infrared radiation penetrates through the material and is converted into heat, and the temperature gradient within the product is reduced in a short period of time. Infrared drying takes place at low temperatures (up to 35 °C) and it helps keep the maximum product quality and natural color. The vegetable drying rate significantly differs depending on the location of the products in relation to the infrared film and product location at the air inlet and outlet. With dried products in 3 parallel shelves the most rapid removal of moisture occurs in the lower shelf (close to the air inlet and film) and the top shelf (close to the air outlet and film). This difference compared to the middle shelf reaches 10–15%. Using the experimental data and multivariate analysis it has been found that the product moisture removal depends on its placement (at the heating film and air inlet, outlet) and the drying time.

**Key words**: IR drying, IR film, carrot, garlic

## INTRODUCTION

Infrared drying (IR) is an effective method of dehydration. It is based on the fact that infrared radiation of certain wavelengths is actively absorbed by water contained in the product. The energy is applied directly to the water of the product, and this is achieved by high efficiency and economy. The absorptivity, the depth of material and the transmissivity are dependent on the IR wave length, density and the properties of the irradiated material.

In the past drying was performed with less emphasis on the quality of the dried products but at the present the quality becomes much more important. Nowadays, we can evaluate the quality of dried products such as texture, color, flavor, nutritional content etc. Drying temperature 40–60 °C is optimal to preserve vitamins, biologically active substances, natural color, flavor and aroma of the dried products. Paakkonen et al. (1999) have shown that IR drying improves the quality of herbs. Pan et al. (2005), Sharma et al. (2005) studied the quality characteristics and advantages of onion infrared drying with different drying temperatures and inlet air velocities. Aboltins and Palabinskis (2016) analyzed three different product (apple, banana slices and grape halves) drying process in the IR film dryer, Prabhanjan et al. (1995) investigated thin layer carrot microwave assisted convective drying,

Carrots *(Daucus carota)* play an important role in human diet because they have one of the highest levels of β-carotene, besides being rich in B vitamins, fiber and

minerals (Prakash et al., 2004). In regard to β-carotene, (Bao & Chang, 1994) cite the carrot as the food with the highest level of this nutrient. The drying of carrot in microwaves has been studied under different aspects. The physical properties and the influence of power ratings on the content of β-carotene was evaluated (Bettega *et al*., 2014), color changes at drying temperatures 50–80 °C was evaluated (Li et al., 2010). Infrared drying characteristic of carrot was investigated in the temperature range of 50–80 °C (Toġrul 2005).

Nowadays, garlic presents a growing interest due to its capacity to form allicin, a powerful antioxidant. Studies have shown that allicin is a natural weapon against infections and is helpful in preventing heart disease and other health disorders (Ankri & Mirelman 1999). Research revealed that drying temperature and thickness of garlic slices significantly affected on color and rehydration ration (Ponciano, 2007).

Dehydration is an important processing operation in the food industry. However, it is well known that the quality of a dehydrated food product is strongly affected by the drying process characteristics (Cui et al., 2003).

Drying can cause changes in the physical properties such as color and structure, as well as the deterioration of aroma compounds or degradation of the nutritional substance reducing the product quality. High drying temperatures reduce the nutrients, aroma, change color, and cause vitamin losses.

The present paper examines the IR film drying possibilities with small heating up to 40 °C. There investigates and compares two products (carrot and garlic slices) drying dynamics depending on the distance from the IR source (film) and the distance from the inlet and outlet of air in the dryer.

## MATERIALS AND METHODS

The experiment was conducted at the Grain Drying and Storage Scientific Laboratory at the Latvia University of Agriculture.

The infrared (IR) dryer (Fig. 1) consisted of a drying chamber (80 x 55 x 75 cm) with a heat source IR film (South Korea EXCEL). The drying equipment body from the inside from all sides was insulated with putupolistorolu and aluminium foil. It increases the drying efficiency by reducing heat losses to the environment. The infrared film with a thickness of 0.338 mm was located on the aluminium foil along the perimeter of the drying chamber. The applied film power was 150 W $m^{-2}$. The maximum temperature on the surface of the film reaches 45 °C.

The drying chamber shelf system can accommodate up to eight shelves, depending on the amount of the drying material (Fig. 2). Shelving is adjustable relative to the upper and lower heating elements and is interconnected with the screw nut. The screw nut adjustment allows for easy shelf height change through various studies. The studies were conducted with three shelf systems and the first plate was located 10 cm from the lower heating element, the second - 35 cm and the third - 60 cm or 15 cm from the upper heater (Fig. 3).

**Figure 1.** IR dryer shelving system.

**Figure 2.** IR dryer with carrot in practice.

Air supply is held by eight adjustable variable cross-section holes that are below the shelf level. The experiments were performed with the fan with a total maximum capacity of 100 $m^3$/h and power 19 W, which is placed on the top of the side wall of the equipment (Fig. 3).

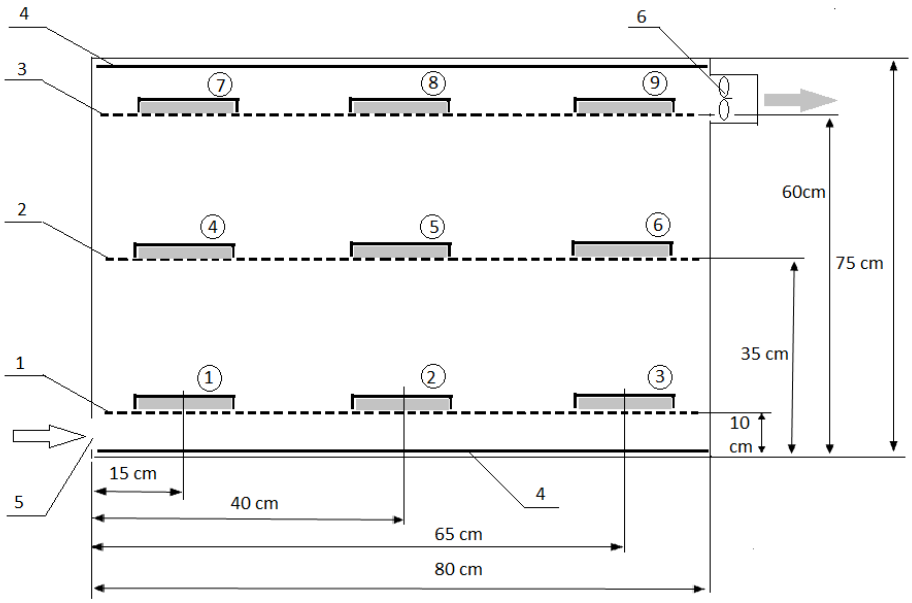



**Figure 3.** Schematic view of IR dryer: 1, 2 and 3 – Shelves; 4 – IR drying film; 5 – Air inlet holes; 6 – Fan.

The carrots were cut into 1 cm thick slices, garlic was cut in half crosswise 1 cm thick (Figs 4 and 5).

The samples were placed on a round drying tray (diameter 20 cm), which consisted of a fine mesh aluminium screen with a plastic frame Fig. 4. These sample plates were put on the drying chamber trays. The trays were placed 10 cm, 35 cm and 60 cm from the IR film on the bottom and 15, 40 and 65 cm from the air inlet (Fig. 3).

**Figure 4.** Garlic cloves before drying.

**Figure 5.** Carrot slices after drying.

The moisture content in the material was identified by gravimetric measurement in time intervals. The samples were weighed on the digital laboratory balance KERN-440-35N with maximum load weight 400 g and with resolution 0.01 g. The total drying time was 24 hours. The initial moisture content of carrot and garlic was 87.2% and 62.1% corresponding.

The average inlet air temperature during the experiment was 18.5 ± 0.5 °C with average humidity 47.3%. The dry matter is determined by laboratory equipment Memmert, drying the product at 102 °C to constant weight of the product.

The experimental data were processed with the program packets MathCad and MatLab.

## RESULTS AND DISCUSSION

Our interest was to find out the carrot and garlic drying efficiency dependence on the position in the drying equipment. Two cases were studied: material moisture dependence on the drying time and the distance from the inlet holes and material moisture dependence on the drying time and the distance from the IR film.

The experimental data show that the outlet air temperature was on average 11 degrees higher than the inlet air temperature during the experiment. Nonlinear multivariable equations between the material moisture M(t, x), drying time t and the distance from the inlet holes at various shelf heights were obtained.

For carrots:

Distance from the IR film on the bottom 10 cm,

$$M(t,x) = 80 - 0.083 \times t + 2.147 \times 10^{-5} \times t^2 + 0.54 \times x - 6.61 \times 10^{-3} \times x^2 + 1 \times 10^{-5} \times t \times x; \quad (1)$$

Distance from the IR film on the bottom 35 cm,

$$M(t,x) = 84.9 - 0.062 \times t + 1.18 \times 10^{-5} \times t^2 + 0.233 \times x - 2.8 \times 10^{-3} \times x^2 + 1.37 \times 10^{-4} \times t \times x; \quad (2)$$

Distance from the IR film on the bottom 60 cm,

$$M(t,x) = 85 - 0.081 \times t + 1.96 \times 10^{-5} \times t^2 + 0.424 \times x - 6.21 \times 10^{-3} \times x^2 - 2 \times 10^{-5} \times t \times x; \quad (3)$$

For garlic:

Distance from the IR film on the bottom 10 cm,

$$M(t,x) = 62.2 - 0.039 \times t + 8.14 \times 10^{-6} \times t^2 - 0.08 \times x - 1.13 \times 10^{-3} \times x^2 + 3.87 \times 10^{-5} \times t \times x; \quad (4)$$

Distance from the IR film on the bottom 35 cm,

$$M(t,x) = 59 - 0.037 \times t + 7.85 \times 10^{-6} \times t^2 + 0.186 \times x - 2.14 \times 10^{-3} \times x^2 + 3.34 \times 10^{-4} \times t \times x; \quad (5)$$

Distance from the IR film on the bottom 60 cm,

$$M(t,x) = 58.6 - 0.042 \times t + 9.9 \times 10^{-6} \times t^2 + 0.169 \times x - 1.94 \times 10^{-3} \times x^2 - 2.7 \times 10^{-5} \times t \times x, \quad (6)$$

where: M(t, x) – material moisture, %; t – drying time, min; x – distance from the air inlet, cm.

The coefficient of determination in these cases was $R^2$ = 0.99. Graphically we compare the carrot and garlic drying dynamics in the case when the products are placed on the top shelf (equations (3) and (6)) Figs 6a and 6b.

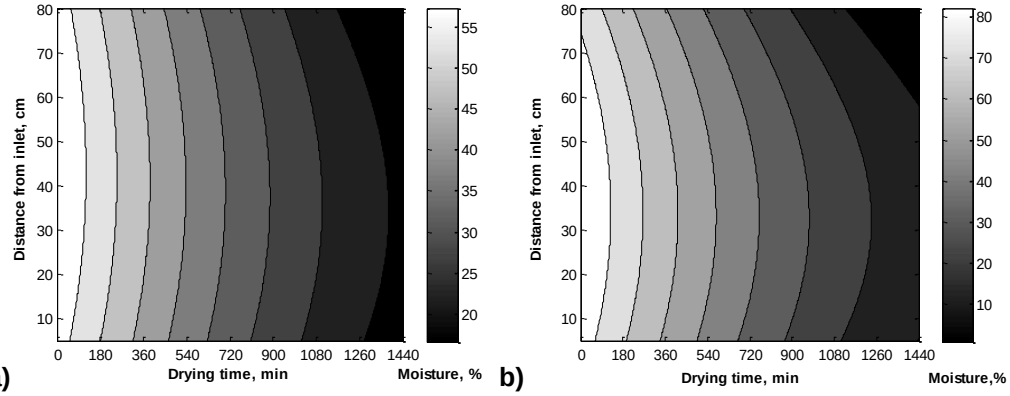



**Figure 6.** Drying time and distance from air inlet influence on **garlic** (a) and **carrot** (b) drying dynamics on top shelf.

As it can be seen, the fan closeness significantly accelerates the drying of the product, especially it is seen at the carrot drying. It can be seen that the air inlet closeness affects the drying rate. This could be explained by a flow of air having less moisture content. The slowest drying took place in the middle of the dryer at all distances of the IR film used in the experiment. As it can be seen from the equations (1)–(6) the drying time is the key parameter that affects the drying dynamics and it is lower in the middle. Its effect in the middle is up to 20% less (for carrots) than at the edges (at the air inlet and outlet) (Fig. 6b).

The experimental results showed that the water removal from the vegetables in the middle shelf was about 10–15% lower than that of the upper and lower shelf.

The distance from the IR film also affect the moisture removal from the material. We tested the effects of 3 different locations, depending on the distance (15, 45, and 65 cm) to the air intake vents. It should be noted that the IR film was on the bottom and top (75 cm from the bottom) in the dryer.

For carrots:

Distance from the air inlet 15 cm,

$$M(t,y) = 81.3 - 0.077 \times t + 1.8 \times 10^{-5} \times t^2 + 0.492 \times y - 6.05 \times 10^{-3} \times y^2 - 5.8 \times 10^{-6} \times t \times y; \quad (7)$$

Distance from the air inlet 45 cm,

$$M(t,y) = 85.8 - 0.061 \times t + 1 \times 10^{-5} \times t^2 + 0.153 \times y - 1.52 \times 10^{-3} \times y^2 - 7.7 \times 10^{-5} \times t \times y; \quad (8)$$

Distance from the air inlet 65 cm,

$$M(t,y) = 86 - 0.089 \times t + 2.5 \times 10^{-5} \times t^2 + 0.28 \times y - 4.22 \times 10^{-3} \times y^2 - 3.6 \times 10^{-5} \times t \times y; \quad (9)$$

For garlic:

Distance from the air inlet 15 cm,

$$M(t,y) = 60 - 0.039 \times t + 8.6 \times 10^{-6} \times t^2 + 0.1 \times y - 1.6 \times 10^{-3} \times y^2 - 2.98 \times 10^{-5} \times t \times y; \quad (10)$$

Distance from the air inlet 45 cm,

$$M(t,y) = 58 - 0.037 \times t + 8.47 \times 10^{-6} \times t^2 + 0.316 \times y - 4.47 \times 10^{-3} \times y^2 - 2.2 \times 10^{-5} \times t \times y; \quad (11)$$

Distance from the air inlet 65 cm,

$$M(t,y) = 59 - 0.035 \times t + 8.58 \times 10^{-6} \times t^2 + 0.245 \times y - 3.6 \times 10^{-3} \times y^2 - 9.4 \times 10^{-5} \times t \times y, \quad (12)$$

where: M(t, y) – material moisture, %; y – distance from the bottom with the IR film, cm.

Graphic comparison of the obtained equations (7)–(12) is shown in Figs 8 and 9.

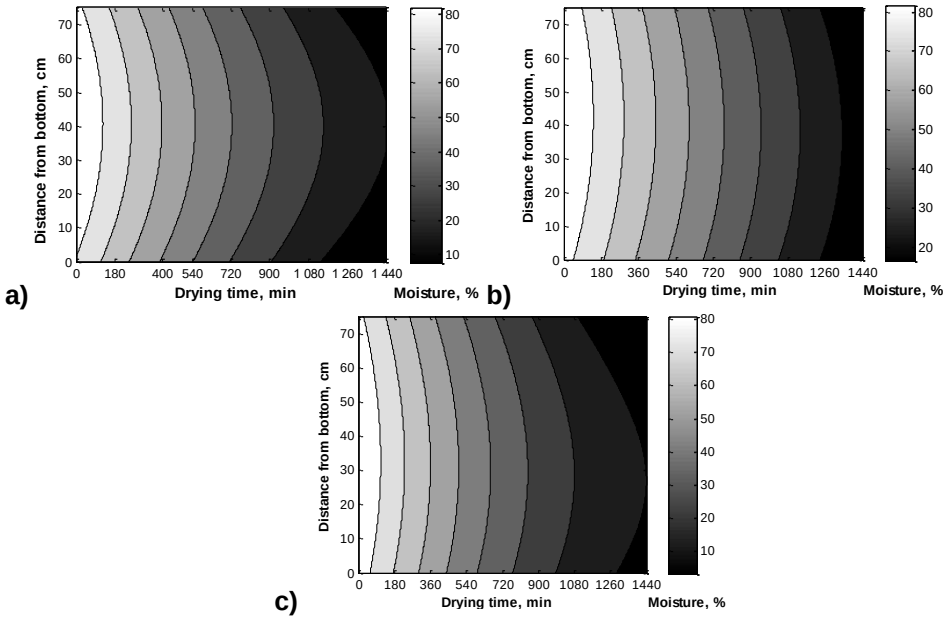



**Figure 8.** Drying time and distance (from IR dryer bottom) influence on water removal from **carrots**: a) 15 cm from air inlet; b) 45 cm from air inlet; c) 65 cm from air inlet.

These graphs show the effect of the direct distance to the film. The fan effect is shown in Figs 8c and 9c. The upper IR film had greater effect on moisture removal. It might be explained by aluminium basis of the trays. For IR rays it is harder to warm the product.

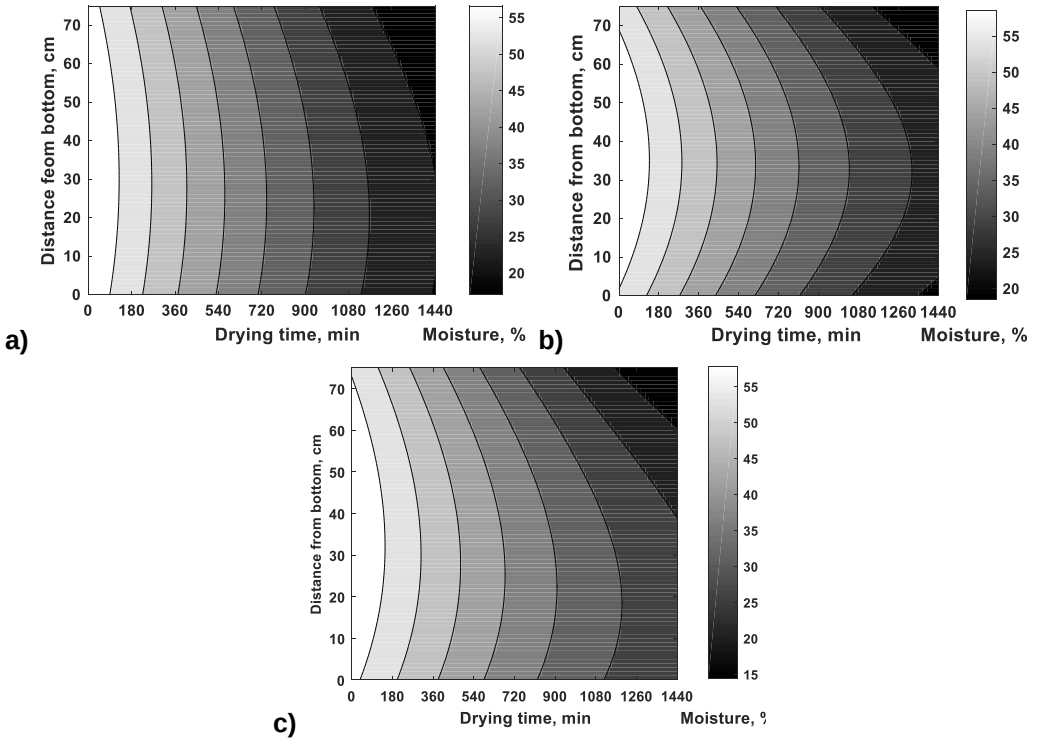



**Figure 9.** Distance (from IR dryer bottom) and drying time influence on water removal from **garlic**: a) 15 cm from air inlet; b) 45 cm from air inlet; c) 65 cm from air inlet.

## CONCLUSIONS

The study shows that drying using the IR film is suitable at low temperatures (up to 35 °C) and it helps to maintain the maximum product quality and natural color. The vegetable drying rate is significantly different depending on the location of the products in relation to the infrared film and product location at the air inlet and outlet. With dried products in 3 parallel shelves it was found that the most rapid removal of moisture occurs in the lower shelf (close to the air inlet and film) and the top shelf (close to the air outlet and film). This difference compared to the middle shelf reaches 10–15%.

## REFERENCES

Aboltins, A. & Palabinskis J. 2016. Fruit drying process investigation in infrared film dryer *Agronomy Research* **14**(1), 5–12.

Ankri, S. & Mirelman, D.1999. Antimicrobial properties of allicin from garlic. *Microbes and Infection* **1**(2), 125–129.

Bao, B. & Chang, K.C. 1994. Carrot juice color, carotenoids, and nonstarchy polysaccharides affected by the processing conditions. *Journal of Food Science* **59**, 1155–1158.

Béttega, R., Rosa J.G., Corrêa R.G., & Freire J.T. 2014. Comparison of carrot (*Daucus carota*) drying in microwave and in vacuum microwave. *Brazilian Journal of Chemical Engineering* **31**(2), 403–412.

Cui, Z.-W.; Xu, S.-Y. & Sun, D.-W. 2003. Dehydration of garlic slices by combined microwave-vacuum and air drying. *Drying Technology* **21**(7), 1173–1184.

Li, Z., Raghavan, G.S.V. & Wang, N. 2010. Carrot volatiles monitoring and control in microwave drying. *LWT - Food Science and Technology* **43**(2), 291–297.

Paakkonen, K., Havento, J., Galambosi, B. & Pyykkonen, M. 1999. Infrared drying of herb. *Agricultural and Food Science in Finland* **8**, 19–27.

Pan, Z., Gabel, M., Amaratunga, S. & Thompson, J.F. 2005. Onion drying using catalytic infrared dryer Report #2, Award No. MR-03-07, 45 p.

Ponciano, S.M. 2007. Optimization of the drying process: an application to the drying of garlic. *Drying technology* **15**(1), 117–136.

Prabhanjan, D.G., Ramaswamy, H.S. & Raghavan, G.S.V. 1995. Microwave-assisted convective air drying of thin layer carrots. *Journal of Food Engineering*, **25**, 283–293.

Prakash, S., Jha, S.K. & Datta, N. 2004. Performance evaluation of dried blanched carrots by three different driers. *Journal of Food Engineering* **62**, 305–313.

Sharma, G.P., Verma, R.C. & Pathare, P.B. 2005. Thin-layer infrared radiation drying of onion slices, *Journal of Food Engineering* **67**, 361–366.

Toǧrul, H. 2005. Suitable drying model for infrared drying of carrot, *Journal of Food Engineering* **77**(3), 610–619.

*Agronomy Research* **15**(S2), 1267–1275, 2017

# The effects of nitrogen rates and intercropping on the occurrence of *Fusarium* spp. on barley kernels

E. Akk[1,*], T.E. Søndergaard[2], J.L. Sørensen[2], H. Giese[2], M.L. Kütt[5], L. Edesi[3], H. Lõiveke[1] and E. Lauringson[4]

[1]Estonian Crop Research Institute, Department of Plant Protection, J. Aamisepa 1, 48309, Jõgeva, Estonia
[2]Aalborg University, Department of Chemistry and Bioscience, Fredrik BajersVej 7H, 9000 Aalborg, Denmark
[3]Estonian Crop Research Institute, Department of Agrotechnology, J. Aamisepa 1, 48309, Jõgeva, Estonia
[4] Estonian University of Life Sciences, Institute of Agricultural and Environmental Sciences, Kreutzwaldi 5, 51014 Tartu, Estonia
[5]University of Vienna, Department of Food Chemistry and Toxicology, Währinger Straβe 38, 1090, Wien, Austria
*Correspondence: elina.akk@etki.ee

**Abstract.** The aim of the field experiments was to compare the effect of nitrogen rates and intercropping on the occurrence of *Fusarium* spp. in barley kernels. The experiments were performed in Central Estonia (58˚33´N, 25˚34´E) in 2009 and 2010. The composition of fungi in spring barley kernels was found through isolation and subsequent sequence analyses of the internal transcribed spacer (ITS) region and morphological features. During the study, 13 species of micro-fungi were identified in the grain samples. The most common species of fungi found in barley were *Fusarium avenaceum, Fusarium poae*, as well as *Phoma pinodella*. The compositions of pathogenic fungi on Estonian barley kernels were affected by the level of nitrogen fertilization and growing on barley-pea intercropping. The study showed tendencies that barley-pea intercropping had fewer occurrences of *Fusarium* species than sole barley.

**Key words:** *Fusarium* spp., spring barley, nitrogen rates, intercropping

## INTRODUCTION

The occurrence of *Fusarium* fungi in small grain crops causes poor quality of the grain and reduction of the yield. The fungal infection of the grain leads to shrunken kernels and contamination with toxic compounds like mycotoxins. The most common disease of cereals is the Fusarium head blight (FHB) caused mainly by five toxic pathogenic species, like *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium poae*, *Fusarium sporotrichioides* and *Fusarium avenaceum* (Champeil et al., 2004; Terzi et al., 2014). The species have been thoroughly studied and investigated worldwide (Wagacha & Muthomi, 2007; Xu et al., 2007; Fernandez et al., 2008; Miedaner et al., 2008; Yli-Mattila, 2010; Fredlund et al., 2013). Moreover, the earlier studies in Estonia have found that the most common diseases on cereals are leaf blights and root rot, following snow

mould and FHB caused by *Microdochium nivale*, *Fusarium culmorum*, *Fusarium avenaceum*, *Fusarium sporotrichiella*, *Fusarium solani*, *Fusarium verticillioides* and *Fusarium oxysporum* (Lõiveke et al., 2004, 2008; Lõiveke, 2008).

The spread of pathogenic soil fungus *Fusarium* spp. is affected by various factors, like agro-meteorological conditions and agronomic practices. Weather conditions like warm or cool temperatures, low or high humidity and rare or intense precipitation during anthesis and maturity of cereals all strongly influence the occurrence of various *Fusarium* strains in different geographical locations (West et al., 2011; Bernhoft et al., 2012; Parikka et al., 2012; Popovski & Celar, 2013). Agronomic practices in the field have also an impact on the diversity and spread of *Fusarium* and other pathogenic fungi on grain. The results of previous studies have found that the preceding crops, fertilization, use of pesticides, crop variety, tillage and cultivation have an effect on the mycobiota in soil, plant and grain. In addition, there are reports that maize as preceding crop, cereals rich rotation, zero or minimal soil tillage and use of glyphosate favoured the spread of *Fusarium* fungi on cereals (Fernandez et al., 2008; Fernandez et al., 2009; Wegulo et al., 2015).

A good method of controlling plant diseases and *Fusarium* spp. is reasonable use of fertilizers in integrated plant production system. Moreover, nitrogen is the most important nutrient affecting disease development in cereal crops (Dordas, 2008). Hauggaard-Nielsen et al. (2008) summarized in the results of their study that intercropping of cereal with pulse improved the use of natural soil nitrogen and reduced the need for mineral nitrogen fertilizers. Cereal mixture with pulse also reduced incidence of the disease in the range of 20–40%. The effect of N fertilizers on occurrence of *Fusarium* spp. is not clear. Van der Burgt et al. (2011) and Krnjaja et al. (2015) reported that, enhancing the rate of N fertilization did not promote the infection of *Fusarium* in grain. On the other hand, Lemmens et al. (2004) and Suproniene et al. (2011) found that high rate of nitrogen increases occurrence of *Fusarium* fungi on grain. Still, little is known how intercropping of cereal with pulse affects the occurrence of *Fusarium* spp.

The aim of the current study was to compare *Fusarium* species composition in spring barley kernels under different rates of mineral nitrogen fertilizer and in pea-barley intercropping.

## MATERIALS AND METHODS

### The field experiment treatment

The field experiments were conducted in 2009 and 2010 on a *Podzoluvisol* (*PD*) (FAO, ISSS, ISRIC. 1998) in Central Estonia (58°33´N, 25°34´E). The experiment was laid out in randomized plots with design of four replicates. Plot size was 2.5 x 10 m. Winter wheat was the previous crop. In the autumn the experimental plots were ploughed. Barley variety 'Anni' and pea variety 'Clarissa' were grown in the experiments. Mineral complex fertilizer (Skalsa N5–$P_2O_5$10–$K_2O$25, dose of fertilizer 250 kg $ha^{-1}$ and nitrogen amount 12.5 kg $ha^{-1}$) was sown together with the seeds for all the treatment plots. The seeds were sown in first decade of May. The ammonium nitrate fertilizer was added as top dressing in the beginning of tillering (GS 21).

The rates of nitrogen (N) were as follows:

1) barley with added ammonium nitrate (dose of fertilizer 312.5 kg $ha^{-1}$) – $N_{120}$ kg $ha^{-1}$;

2) barley with added ammonium nitrate (dose of fertilizer 138 kg $ha^{-1}$) – $N_{60}$ kg $ha^{-1}$;
3) barley without added ammonium nitrate – $N_{40}$;
4) barley-pea intercrops without ammonium nitrate – BP.

We presumed that the soil of barley-pea intercrop contains naturally around 40–50 kg of free N per ha (Freyer, 2003). The sowing rate of the sole barley was 550 seeds per $m^2$ and in the intercrop 120 barley and 80 pea seeds per $m^2$. Herbicide Butoxone (active ingredient MCPB 400 g $l^{-1}$) was used for weed control in dosage of 3.8 l $ha^{-1}$, and no fungicides were used for disease control. After harvesting in the end of August the seeds were dried to 14% of standard moisture, sorted and a 1.5 kg sample was taken from each treatment. Samples were stored at –4 °C until laboratory analysis.

**The isolation and identification of fungi**

For isolation and identification of fungi the surface of barley grains were sterilized for 5 minutes with 1% of sodium hypochlorite (NaOCl) and rinsed twice with sterile distilled water. According to Abildgren et al. (1987), the 100 kernels were placed in Petri dishes containing CZID medium (35 g of Czapek Dox broth, 15 g of Agar, 1 ml of trace solution, 1 ml of dichloran, 1 ml of Chloramphenicol in 1 L of MQ water, autoclaved at 121 °C for 15 min). The plates were incubated at 20 °C in the dark (8 h) and in the light (16 h) cycle. After seven days of incubation, the fungi were transferred to Potato Dextrose Agar (PDA: 39 g of Potato Dextrose Agar, 1 mL of trace solution, 1 L of MQ water, autoclaved at 121 °C for 15 min) and the inoculated Petri dishes were kept in the dark for 14 days at 28 °C. Fungi determined morphologically accordingly to Samson et al. (2002) and Leslie and Summerell (2006). After incubation the DNA of all fungi was purified with the Ultra Clean Microbiol DNA Isolation Kit (MOBio Inc.CA) according to the manufacturer´s protocol. The internal transcribed spacer (ITS) region of the rDNA gene (S) was amplified using fungal universal primers: ITS1 (5´TCCGTAGGTGAACCTGCGG) and ITS4 (5´TCCTCCGCTTATTGATATGC) (White et al. 1990). The thermal cycler profile was used with denaturation at 98 °C for 5 min; followed by 30 cycles of amplification with denaturation at 98 °C for 1 min, primer annealing at 52 °C for 1 min, extension at 72 °C for 5 min, followed by cooling at 4 °C. Next, the 35 µl of PCR product was taken and cleaned further with Qiaquik PCR Purification Kit (Qiagen). The Sample Submission guide of EUROFINS MWG Operon was followed to prepare DNA to sequence the PCR products. The obtained DNA sequences were analysed using the UNITE database (https://unite.ut.ee) (Koljalg et al., 2013). The BLAST searching system at GenBank sequence database http://blast.ncbi.nlm.nih.gov/ was used to identify the fungi strains.

**Weather conditions**

The weather conditions during vegetation period (from May to August) in 2009 and 2010 were different (Table 1). In 2009, the average temperature for four-month period was 2.4 °C degrees cooler than in 2010. The overall amount of precipitation over the years did not differ significantly, being 332.9 mm in 2009 and 316.2 mm in 2010. However, the amount of precipitation during the vegetation period distributed unevenly. In 2009, June was rainy (sum of precipitation 85.3 mm) and July extremely rainy (sum of precipitation 135.6 mm). In 2010, July was dry (sum of precipitation 42.8), but August was very rainy (sum of precipitation 143.2 mm).

**Table 1.** Weather conditions in 2009 and 2010 in Central Estonia

| | | Day of rainfall | | Sum of precipitation, mm | | Average air temperature, °C | |
|---|---|---|---|---|---|---|---|
| Month | Decade | 2009 | 2010 | 2009 | 2010 | 2009 | 2010 |
| May | I | 3 | 7 | 5.2 | 38.2 | 10.5 | 7.3 |
| | II | 2 | 4 | 6.0 | 4.8 | 9.9 | 17.2 |
| | III | 3 | 5 | 5.6 | 9.8 | 13.9 | 12.5 |
| | | **8** | **16** | **16.8** | **52.8** | **11.4** | **12.3** |
| June | I | 4 | 3 | 48.7 | 18.6 | 11.3 | 13.9 |
| | II | 9 | 6 | 35.6 | 27.4 | 13.1 | 13.8 |
| | III | 2 | 2 | 1.0 | 31.4 | 17.3 | 16.3 |
| | | **15** | **11** | **85.3** | **77.4** | **13.9** | **14.7** |
| July | I | 5 | 3 | 46.2 | 16.0 | 16.1 | 20.6 |
| | II | 6 | 3 | 47.6 | 9.2 | 17.9 | 23.6 |
| | III | 8 | 4 | 41.8 | 17.6 | 17.4 | 22.8 |
| | | **19** | **10** | **135.6** | **42.8** | **17.1** | **22.3** |
| August | I | 5 | 3 | 20.6 | 10.6 | 17.0 | 21.4 |
| | II | 7 | 6 | 56.6 | 68.4 | 15.2 | 19.8 |
| | III | 4 | 7 | 18.0 | 64.2 | 15.0 | 14.6 |
| | | **16** | **16** | **95.2** | **143.2** | **15.7** | **18.6** |
| Total/average | | **58** | **53** | **332.9** | **316.2** | **14.6** | **17.0** |

**The statistical analysis**

The frequency of occurrence isolated fungal species calculated as number of isolated species divided total number of isolated species. The percentage occurrence of fungal species was found as the number of kernels in what species occurred divided on total number of kernels multiplied by 100. Analysis of variance (ANOVA, SAS 2002) used for determining effects of nitrogen rates and intercropping on the occurrence of *Fusarium* spp. on the barley kernels. The significance threshold was set at $p < 0.05$.

## RESULTS AND DISCUSSION

During the study in total 85 isolates and 12 species of fungi were identified in the spring barley kernels, while the occurrence of *Fusarium* fungi was appreciable different in both years of the study (Table 2). The seven fungal genera were identified in 2009 and two fungal genera in 2010. In 2009, *Fusarium* spp. were isolated from 38% of kernels, whereas the occurrence of other pathogenic fungi was 62%. While in 2010, *Fusarium* was the common genus (96%) isolated from barley kernels. Our results confirm the findings of several researchers: that the weather factors have a significant effect on the occurrence of *Fusarium* spp. and other pathogenic fungi in kernels (Blandino et al., 2008; Parikka et al., 2012; West et al., 2012). Research of Blandino et al. (2008) showed that the climatic factors influenced the presence of fungal species; moreover, the rate of nitrogen application had an effect on fungal infection in maize kernels. Parikka et al. (2012) suggested that warm weather with more rainfall near harvest increases the spread of *Fusarium* spp., while high temperature, dryness and intense rainfall cause plant stress and favour of *Fusarium* infection on cereals in Northern Europe. West et al. (2012) concluded that the weather conditions together with the effect of agronomic practices influenced the interaction of pathogenic fungal species. Consequently, our study confirmed that warmer vegetation period and rainy harvesting season as it was in 2010

favoured the contamination of kernels with *Fusarium* fungi. On the contrary, in 2009, the moderate air temperatures with high rainfalls in July increased the occurrence of other pathogenic fungi that may suppress *Fusarium* fungi.

**Table 2.** Frequency of occurrence (%) of isolated fungal species from spring barley kernels in 2009 and 2010

| Fungal species | Frequency (%) | | |
|---|---|---|---|
| | 2009 | 2010 | Mean |
| *Arthrinium sacchari* | 2.8 | 2.0 | 2.4 |
| *Alternaria infectoria* | 2.8 | 0.0 | 1.2 |
| *Epicoccum nigrum* | 8.3 | 0.0 | 3.5 |
| *Fusarium avenaceum* | 11.1 | 51.0 | 34.1 |
| *Fusarium equiseti* | 16.7 | 2.0 | 8.2 |
| *Fusarium oxysporum* | 0.0 | 6.1 | 3.5 |
| *Fusarium poae* | 2.8 | 16.3 | 10.6 |
| *Fusarium sporotrichioides* | 0.0 | 10.2 | 5.9 |
| *Fusarium tricinctum* | 5.6 | 10.2 | 8.2 |
| *Microdochium bolleyi* | 13.9 | 2.0 | 7.1 |
| *Phoma pinodella* | 22.2 | 0.0 | 9.4 |
| *Parastagonospora nodorum* | 13.9 | 0.0 | 5.9 |

Unlike the weather condition, the impact of nitrogen on the occurrence of *Fusarium* and other pathogenic fungi was less obvious. However, we still found that the effects of N rates and intercropping were statistically significant. In this study, the occurrence of micro-fungi on the barley kernels was higher at $N_{120}$ and $N_{40}$ compared to $N_{60}$ rate and intercropping treatment (Fig. 1). The frequency of occurrence of micro-fungi on the barley kernels in the intercropping (BP) treatment was 1.8 times lower than in $N_{40}$ and 1.6 times lower than in $N_{120}$ treatment. Several researchers concluded that high rates of nitrogen fertilizer application increased *Fusarium* infection level in grain (Suproniene et al., 2012; Lemmens et al., 2004, van der Burgt et al., 2011). Lemmens et al. (2004) explained that increasing N input changes the plant canopy density, which in turn influences microclimatic conditions in plant-soil environment and delays the flowering period, therefore creating favourable conditions for infection. Probably the low or high rate of nitrogen leads the plants to stress that makes them more susceptible to *Fusarium* spp. and other pathogenic micro-fungi (Blandino et al., 2008). The highest occurrence of micro-fungi in the rate of N40 was probably due to the stress caused by nutrient deficiency; also, the plant height (unpublished data) was lower than in other treatments. It may be caused by the fact that the macro-conidia from Fusarium spp. can splash-disperse as high as 40–60 cm vertically from the source (Jenkinson & Parry, 1994; Lemmens et al., 2004), thereby contaminating the barley kernels. Review article by Dordas (2008) discusses the effect of N on contamination of pathogen depending on the type of fungi. It says that although high N application increases the severity of infestation by obligate parasitic fungi (such as Puccinia spp. and other diseases), it also decreased the infestation by facultative fungi (such as Alternaria spp., Fusarium spp.) In addition to interactions, repression and competitions are held between the different microorganisms, where the host plant responds with a complex of biochemical reactions, thereby becoming more susceptible or resistant to the pathogens (Dordas, 2008). The characteristic of plant density can also influence a distribution of *Fusarium* and other

fungi whereas in tight plant spacing humid conditions could remain for longer than in a sparser intercrop. In this study, we found tendencies that the high occurrence of *Fusarium* fungi corresponds with low rate of the $N_{40}$, followed the top dressing treatment rates of the $N_{120}$ and $N_{60}$.

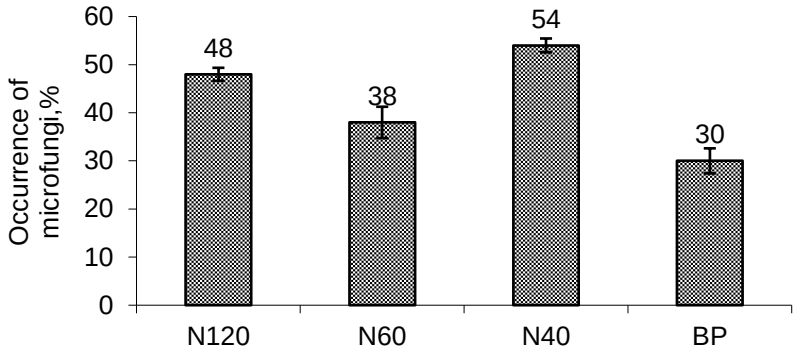



**Figure 1.** The average frequency of occurrence of micro-fungi in barley cropped under different nitrogen levels and barley-pea (BP) intercropping in 2009 and 2010.

Our study revealed that *Fusarium avenaceum* and *Fusarium poae* were the most common fungal species isolated from barley kernels (Table 3). *Phoma pinodella* and *Microdochium bolleyi* had high frequency of other isolated fungi. *Fusarium lateritium* dominated in kernels from all the different rates of nitrogen and also in intercropping. *Fusarium poae* was detected in the kernels from the mineral nitrogen rate and the rate of $N_{40}$ but not in intercropping barley kernels. *Fusarium sporotrichioides* identified only in kernels from the nitrogen rate of $N_{120}$ and $N_{40}$, but not in $N_{60}$ and intercropping kernels. In barley kernels from intercropping treatment we identified only *Fusarium lateritium* compared at the rates of $N_{120}$, $N_{60}$ and $N_{40}$. Other identified micro-fungi also showed interesting tendencies. The pathogenic fungi *Parastagonospora nodorum* occurred mainly in the rate of $N_{120}$ in barley kernels. It was statistically significant that pathogenic fungus *Phoma pinodella* dominated only in kernels from intercrop. However, our results showed that the intercropping of cereal with pea might be an option to decrease the infestation of barley grain with *Fusarium* fungi. In addition, the occurrence of micro-fungi on grain decreased in moderate mineral nitrogen application as $N_{60}$ compare with high rate and very low rate of nitrogen.

In this study the species *Fusarium avenaceum* dominated in the kernels from all treatments. The *Fusarium* species like *Fusarium poae* and *Fusarium sporotrichioides* were modestly represented in top dressing mineral nitrogen type and $N_{40}$ but not in intercropping.

Results from this study showed tendencies that in the barley kernels from intercropping treatment, *Fusarium* species occurred less than in mineral nitrogen top dressing fertilization treatment. Likewise van der Burght et al. (2011) found significant positive effect of top dressing on the FHB infected seeds. We consider that compared to the intercropping of barley with pea the top dressing at sole barley favourably affected the grain contamination by *Fusarium* and in low fertilization rate as $N_{40}$ caused stress in

plants, thereby making them more susceptible to the *Fusarium* and other pathogenic fungi. Hauggaard-Nielsen et al. (2001) explained that in intercropping the barley is capable to take more soil mineral nitrogen in contrast to sole barley. This explains why barley mixed with pea does not starve from lack of nitrogen like barley in the $N_{40}$ treatment. Thus, lower occurrence of *Fusarium* spp. in intercropped kernels compared with other types of treatments may be due to moderate supply of nitrogen as well as sparse plant density in this treatment.

**Table 3.** The probability of occurrence of fungi species in barley cropped under different rates of nitrogen and barley-pea (BP) intercropping in 2009 and 2010

| Species | $N_{120}$ | $N_{60}$ | $N_{40}$ | BP |
|---|---|---|---|---|
| *Arthrinium sacchari* | ns | no | ns | no |
| *Alternaria infectoria* | ns | ns | ns | no |
| *Epicoccum nigrum* | ns | no | ns | no |
| *Fusarium avenaceum* | 0.004 | 0.001 | 0.002 | 0.006 |
| *Fusarium equiseti* | ns | ns | ns | ns |
| *Fusarium oxysporum* | ns | no | ns | no |
| *Fusarium poae* | 0.004 | 0.05 | 0.04 | no |
| *Fusarium sporotrichioides* | 0.05 | no | 0.004 | no |
| *Fusarium tricinctum* | ns | no | ns | ns |
| *Microdochium bolleyi* | ns | ns | ns | no |
| *Phoma pinodella* | no | no | no | 0.002 |
| *Parastagonospora nodorum* | 0.001 | no | ns | no |

no – not occurred

ns – not statistically significant at the $p < 0.05$

## CONCLUSIONS

Based on the results of study we found tendencies that the higher air temperature and rainy weather during the maturity of barley favourably influenced the infestation ofkernels by *Fusarium* spp. The moderate temperatures and more than average precipitation in 2009 were favourable conditions for *Phoma pinodella*, *Microdochium bolleyi* and other mould. Frequency of occurrence of *Fusarium* was 36% in 2009. In warmer and rainy 2010 *Fusarium* species were most abundant (frequency of occurrence of *Fusarium* 96%) fungi in the barley.

The barley-pea intercropping and investigated rates of mineral nitrogen in barley influenced the occurrence of *Fusarium* and other micro-fungi differently.

The *Fusarium avenaceum* was dominant in all treatments. Experiments revealed tendencies that *Fusarium poae* and *Fusarium sporotrichioides* were mainly represented in the mineral fertilizer top dressing treatments. The frequency of occurrence of micro-fungi in the intercropping treatment was lower as mineral nitrogen fertilizer treatments.

*Fusarium* spp previously not been investigated in kernels from intercropping and nd under two years experiments not enough to make definitive conclusions. We found interesting tendencies, but more experiments need for final conclusions.

ACKNOWLEDGEMENTS. Financial support for this research was provided by the Estonian Ministry of Agriculture. The authors would like to thank the colleagues from Aarhus and Aalborg Universities for the design of laboratory experiments.

## REFERENCES


Abildgren, M.P., Lund, F., Thrane, U. & Elmholt, S. 1987. Czapek-Dox agar containing iprodione and dicloran as a selective medium for the isolation of *Fusarium* species. *Letters in Applied Microbiology* **5**, 83–6.

Bernhoft, A., Torp, M., Clasen, P-E., Løes A-K. & Kristoffersen, A.B. 2012. Influence of agronomic and climatic factors on *Fusarium* infestation and mycotoxin contamination of cereals in Norway. *Food Additives and Contaminants* Part A **29**, 1129–1140.

Blandino, M., Reyneri, A. & Vanara, F. 2008. Influence of nitrogen fertilization on mycotoxin contamination of maize kernels. *Crop Protection* **27**, 222–230.

Champeil, A., Fourbet, J.F., Doré, T. & Rossignol, L. 2004. Influence of cropping system on Fusarium head blight and mycotoxin levels in winter wheat. *Crop Protection* **23**, 531–537.

Dordas, C. 2008. Role of nutrients in controlling plant diseases in sustainable agriculture. A review. *Agronomy for Sustainable Development* **28**, 33–46.

FAO, ISSS, ISRIC.1998. World reference base for soil resources. *World Soil Resources Report*. Rome, 84.

Fernandez, M.R., Huber, D., Basnyat, P. & Zentner, R.P. 2008. Impact of agronomic practices on populations of *Fusarium* and other fungi in cereal and noncereal crop residues on the Canadian Prairies. *Soil and Tillage Research* **100**(1–2), 60–71.

Fernandez, M.R., Zentner, R.P., Basnyat, P., Gehl, D., Selles, F. & Huber, D. 2009. Glyphosate associations with cereals diseases caused by *Fusarium* spp. in the Canadian Praires. *European Journal of Agronomy* **31**, 133–143.

Fredlund, E., Gidlund, A., Sulyok, M., Börjesson, T., Krska, R., Olesen, M. & Lindblad, M. 2013. Deoxynivalenol and other selected *Fusarium* toxins in Swedish oats – Occurrence and correlation to specific Fusarium species. *International Journal of Food Microbiology* **167**, 276–283.

Freyer, B. 2003. Fruchtfolgen.Konventionell.Integriert.Biologisch.Verlag Eugen Ulmer.

Hauggaard-Nielsen, H., Ambus, P. & Jensen, E.S. 2001. Interspecific competition N use and interference with weeds in pea-barley intercropping. *Field Crops Research* **70**, 101–109.

Hauggaard-Nielsen, H., Jørnsgaard, B., Kinane, J. & Jensen, E.S. 2008. Grain-legume cereal intercropping: The practical application of diversity, competition and facilitation in arable and organic cropping system. *Renewable Agriculture and Food Systems* **23**, 3–2.

Jenkinson, P. & Parry, D.W. 1994. Splash dispersal of conidia of *Fusarium culmorum* and *Fusarium avenaceum*. *Mycological Research* **98**, 506–510.

Koljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L, Taylor, A.FS, Bahram, M., Bates, S., Bruns, T.M., Douglas, B., Drenkhan, T., Eberhardt, U., Daeñas, M.,Grenenc, T., Griffith, G.W., Hartmann, M., Kirk, P.M., Kohout, P., Larsson, E., Lindahl, B.D., Lücking, R., Martin, M.P., Matheny, B.P.,Nguyen, N.H., Niskanen, T., Oja, J., Peay, K., Peintner, U., Peterson, M., Poldmaa, K., Saag, L., Saar, J., Schüβler, A., Scott, J., Senes-Guerro, C., Smith, M.E., Suija, A., Taylor, D.L., Telleria, M.T., Weiss, M. & Larsson, K-H. 2013. Towards a unified paradigm for sequence - based identification of fungi. *Molecular Ecology* **22**, 5271–5277.

Krnjaja, V., Mandić, V., Lević, J., Stanković, S. & Petrović, T. 2015. Influence of N-fertilization on Fusarium head blight and mycotoxin levels in winter wheat. *Crop Protection* **67**, 251–256.

Lemmens, M., Haim, K., Lew, H. & Ruckenbauer, P. 2004. The Effect of Nitrogen Fertilization on Fusarium Head Blight Development and Deoxynivalenol Contamination in Wheat. *Journal of Phytopathology* **152**, 1–8.

Leslie, J.F. & Summerell, B. A. 2006. The *Fusarium* Laboratory Manual. Blackwell Publishing.

Lõiveke, H., Ilumäe, E. & Laitamm, H. 2004. Microfungi in grain and grain feeds and their potential toxicity. *Agronomy Research* **2**, 195–205.

Lõiveke, H. 2008.Teraviljadefusarioosid Eestis. Summary 5–6.
Lõiveke, H., Ilumäe, E. & Akk, E. 2008. Microbiology and safety of grain. *Journal of Agricultural Science* **19**(2), 38–44. (in Estonian, abstract in English).
Miedaner, T., Cumagun, J.R. & Chakraborty, S. 2008. Population Genetics of Three Important Head Blight Pathogens *Fusarium graminearum*, *F. pseudograminearum* and *F. culmorum*. *Journal of Phytopathology* **156**, 129–139.
Parikka, P., Hakal, K. & Tiilikkala, K. 2012. Expected shifts in *Fusarium* species' composition on cereal grain in Northern Europe due to climate change. *Food Additives and Contaminants* Part A **29**, 1543–1555.
Popovski, S. & Celar, F.A. 2013. The impact of agro-environmental factors on the infection of cereals with *Fusarium* species and mycotoxin production-a review. *Acta Agriculturae Slovenica* **101**, 105–116.
Samson, R.A., Haekstra, E.S., Frisvad, J.C. & Filtenberg, O. 2002. Introduction to Food- and Airborne Fungi. Sixth edition. Centraalbureau Voor Schimmelcultures – Utrecht.
Suproniene, S., Mankevičiene, A., Kadziene, G., Feiziene, D., Feiza, V., Semaskiene, R. & Dabkevicius, Z. 2011. The effect of different tillage-fertilization practices on the mycoflora of wheat grains. *Agricultural and Food Science* **20**, 312–326.
Suproniene, S., Mankevičiene, A., Kadziene, G., Kačergius, A., Feiza, V., Freiziene, D., Semaškiene, R., Dabkevičius, Z. & Tamošiūnas, K. 2012. The impact of tillage and fertilization on *Fusarium* infestation and mycotoxin production in wheat grain. *Žemdirnyste=Agriculture* **99**, 265–272.
Terzi, V., Tumino, G., Stanca, AM. & Morcia, C. 2014. Reducing the incidence of cereal head infection and mycotoxins in small grain cereal species. *Journal of Cereal Science*, 284–293.
Van der Burgt, G.J.H.M., Timmermanns, B.G.H., Scholberg, J.M.S. & Osman, A.M. 2011. *Fusarium* head blight and deoxynivalenol contamination in wheat as affected by nitrogen fertilization. *Wageningen Journal of Life Sciences* **58**, 123–129.
Wagacha, J.M. & Muthomi, J.W. 2007. *Fusarium culmorum*: Infection process, mechanisms of mycotoxin production and their role in pathogenesis in wheat. *Crop Protection* **26**, 877–885.
Wegulo, S.N., Baenziger, P.S., Nopsa, J.H., Bockus, W.W. & Hallen-Adams, H. 2015. Management of *Fusarium* of head blight of wheat and barley. *Crop Protection* **73**, 100–107.
West, J.S., Holdgate, S., Townsend, J.A., Edwards, S.G., Jennings, P. & Fitt, B.D.L. 2012. Impacts of changing climate and agronomic factors on fusarium ear blight of wheat in the UK. *Fungal Ecology* **5**, 53–61.
Xu, X., Nicholson, P. & Ritieni, A. 2007. Effects of fungal interactions among *Fusarium* head blight pathogens in disease development and mycotoxin accumulation. International *Journal of Food Microbiology* **119**, 67–71.
Yli-Mattila, T. 2010. Ecology and Evolution of toxigenic *Fusarium* species in cereals in northern Europe and Asia. *Journal of Plant Pathology* **92**(1), 7–18.

*Agronomy Research* **15**(S2), 1276–1286, 2017

# Development of gluten-free bread with unconventional raw ingredients of high nutritional value and antimicrobial activity

N. Dubrovskaya[1,*], O. Savkina[2], L. Kuznetsova[2] and O. Parakhina[3]

[1]Peter the Great St.Petersburg Polytechnic University, Polytechnicheskaya, 29, 195251, St.Petersburg, Russia, Russian Federation,
[2]Institute of Refrigeration and Biotechnologies, ITMO University, Lomonosova street, 9, 191002, St.Petersburg, Russia, Russian Federation
[3] St.Petersburg branch State Research Institute of Baking Industry, Podbelskogo highway 7, 196608, St.Petersburg, Pushkin, Russia, Russian Federation
*Correspondence: dubrovskaja_nata@mail.ru

**Abstract.** Two types of rowan powder *(*botanical species *Sorbus aucuparia)* as unconventional raw ingredients of high nutritional and biological value as well as three types of dietary fiber and pectin were used in development of gluten-free bread. These raw ingredients have high water-holding capacity and a rich biochemical composition that makes it possible to use them not only as thickeners and structure forming agents, but also as enriching additives. It was experimentally found that the citrus fiber and pectin improved the bread specific volume and the crumb compressibility if compared to the control sample. The content of vitamins A, E, PP, C, B complex, minerals - iron, magnesium, calcium, potassium, selenium, organic acids, including preservatives rendering action (citric, lactic, sorbic, benzoic) were found in rowan powder. It was found that rowan powder and citrus fiber had a significant effect on the increase in the content of dietary fiber in 2.5–5.4 fold and iron in 2–3.5 fold. The content of dietary fiber in bread with 4% of rowan fruit powder is 3.6 g $100g^{-1}$ while in bread with 8% rowan pomace it is 4.3 g $100g^{-1}$, which corresponds to the dietary fiber daily needs satisfaction respectively by 10.0% and 21.5%. It was found that bread with rowanberry powder had 66.7% more water-soluble antioxidants. The contamination of the main gluten-free raw material (soy protein, rice flour and corn starch extrusion and corn, rowan powder) and its influence on ropy disease of gluten free bread were established. Four spore forming bacteria strains were isolated from gluten-free raw materials and its ability to cause ropy disease of gluten free bread was proved. It was also found that using of rowan powder slow down ropy disease and mold spoilage due to the organic acids in its composition and the bread acidity increase.

**Key words:** Gluten-free bread, rowan powder, quality, nutritional value, antioxidant activity, microflora, microbial resistance

## INTRODUCTION

Due to environment adverse effects, genetic and allergic diseases growth, the dietary and functional products development is one of main food industry objectives. Today bakery products traditionally form the basis of the population diet both in Russia and Europe being the most consumed grain foods. Therefore, in their production special attention is paid to the issues of quality, safety and assortment of gluten-free bread due

to the wide variety of food ingredients that provide the human body with the necessary physiologically active substances. The improved diagnostic methods have led to the identification of one of the 21st century genetic diseases – celiac enteropathy (coeliac disease). It is a serious genetic autoimmune disorder where gluten ingestion leads to chronic inflammation of the mucous membrane with complete atrophy of the intestinal villi. Coeliac disease is caused by a reaction to gluten that is a protein found in wheat and other grains such as barley, rye, triticale and oats in the early stages (Troncone & Jabri, 2011). At present there is no cure and the only effective treatment for this disease is a lifelong gluten-free diet. It leads to the intestinal mucosa recovery, improves symptoms, and reduces risk of developing complications in most people (Rashtak & Murray, 2012). Bread is included into the daily diet of all population segments of Russia. But a large group of the population of Russia suffering from a celiac disease is forced to abandon the traditional bread consumption and to start consuming gluten-free products (Vokhmyanin, 2009). Therefore, the demand for gluten-free products, produced from natural gluten-free raw materials (rice, corn, buckwheat flour, corn starch and other gluten-free cereal, soy protein, lupine, amaranth, legumes, etc.) is constantly growing. However, existing gluten-free products have a number of disadvantages, namely: low nutritional value, mild flavor and odor, susceptibility to microbiological spoilage (Thompson, 2000; Hager et al., 2012; Javaria et al., 2016). This is due to the fact that for the production of gluten-free products, including bread, raw materials with low nutritional value are mainly used (do Nascimento, 2013; Pellegrini & Agostoni, 2015). Gluten-free bread does not have traditional odor of freshly baked bread and does not satisfy consumers' flavor demands (Pszczola, 2012). In addition, gluten-free bread stales quickly because of the high content of starch in the recipe. The Russian gluten-free bread rapidly undergoes microbial spoilage because it has very low acidity (pH above 6). Today a lot of scientists and food industry specialists are developing ways of improving gluten-free bread quality (Krasilnikov et al., 2013; Nachay, 2013; Kuznetsova & Dubrovskaya, 2014; Matos & Rosell, 2014).

The aim of research is to develop gluten-free bread using unconventional raw ingredients of high nutritional and biological value – dietary fiber and pectin, as well as the rowan powder (botanical species *Sorbus aucuparia*). These ingredients have high water-holding capacity and a rich biochemical composition that makes it possible to use them not only as thickeners and structure forming agents, but also as enriching additives. The effect of non-traditional raw ingredients on biotechnological processes in the dough and on the quality, freshness, structural and mechanical properties of the crumb, nutritional value, antioxidant activity and resistance to mold growth and the ropy disease of gluten-free bread with fiber and pectin and rowan powder were studied.

## MATERIALS AND METHODS

Two types of rowan powder (botanical species - *Sórbus aucupária*) – the ordinary rowan fruit powder and the powder from the pomace of the fruits of rowan varietal phoenicea – were used in this research. The ordinary rowan is characterized by bitterness and astringency of the fruit. The rowan varietal phoenicea, a new variety grown in the Michurin All-Russian Research Institute of genetics and breeding of fruit plants, has, as

its distinctive feature, the lack of bitterness and astringency and high content of biologically active substances, especially vitamins, micro- and macroelements.

Acidity, content of volatile acids and organic acids (malic, succinic, sorbic etc., including fatty and phenolic acids), vitamins, and minerals were determined in the rowan powder. Gluten-free bread was evaluated by organoleptic and physico-chemical parameters such as moisture, acidity, porosity and specific volume.

Acidity was determined by titration, using 0.1 n. solution of NaOH. Content of volatile acids was determined by neutralization of evaporated volatile acid using 0.1 n. solution of NaOH.

Analysis of biochemical composition of the rowan powder was carried out using gas-liquid chromatography with mass spectrometry (GC-MS) on a chromatograph 'Agilent 6850' (USA). Antioxidant activity of rowanberry powder and gluten free bread was determined using amperometric method.

As thickeners and the texture forming substances the citrus fiber Citri-Fi 100, Citri-Fi 200, Herbacel AQ Plus and citrus pectin (a Genu pectin type BIG) were studied. Corn extrusion starch was used as control.

Water holding capacity of the thickener is studied in the following way. 5 g of the thickener were mixed with 30 ml of water (or more), then it was mixed for 1 min at 1000 rpm, left at rest for 30 min, centrifuged for 25 min with the imminent 3200 rpm, feeding the separated water. The increased water-holding capacity was calculated according to the ratio of the mass of released water to the initial weight and multiplied by 100%

Optimization of doses of rowan powder in the recipe was carried out by a laboratory test baking. The formulation of control bakery products was: bakery mix total 100% (including corn starch – 57.8%, extrusion corn starch – 10.0%, rice flour – 19.7%, soy protein – 9.7%, salt – 0.8%, sugar – 2.0%) and yeast – 2.5%, vegetable oil – 4.0%.

The enrichment of bakery products is achieved by adding rowan powder instead of corn starch. The content of additive varied from 2 to 10% with the increment of 2 %. All test samples were prepared by using the straight dough method. Test bakery products weighing 250 g were baked in laboratory at the temperature of 210 °C during 18 min.

The assessment of bakery products quality was carried out by following properties: Organoleptic – appearance (shape, surface, crumb color), condition of crumb (porosity and texture), flavor ad odor; physico-chemical and physical - mass proportion of moisture was determined by drying at a temperature of 130 °C during 45 minutes, acidity was determined by titration, using 0.1 n. solution of NaOH, porosity was determined as the ratio of pore volume to the total volume of products, pore volume – as the difference between the volume of product and the volume of non-porous mass, specific volume – as the ratio of product volume to 100 g of bread. The contents of the spore-forming bacteria number was determined by plating the heated samples of meat-peptone agar. It was suspended in 100 $cm^3$ of sterile water and heated in a water bath for 10 minutes at a temperature of 90–94 °C in order to inactivate the vegetative cells of 10 g of the studied raw materials. The prepared dilutions $10^{-1}$–$10^{-2}$. 1 ml of each dilution were added to Petri dish and flooded with 10 ml mycopathologia agar, and then for 2 days were cultured in a thermostat at 37 °C (Afanasjeva, 2003).

To determine the ability of the isolated spore-forming bacteria that cause ropy disease a bread crumb with the spores was prepared. Thus, the spore-forming bacteria on meat-peptone medium was added to the surface of the sliced gluten free bread and cultured at a temperature of 37 °C for 96 hours or until signs of disease. The diseased

bread was dried in an oven at a temperature of 50 ± 2 °C and milled to obtain crumbs. 1% of infected crumbs were added while kneading the dough for gluten-free bread. The ready bread was stored at 37 °C until the appearance of ropy disease symptom.

## RESULTS AND DISCUSSION

Gluten-free bread is characterized by a low content of vitamins, minerals and fiber, bland, and empty flavor, odor is not enough pronounced (Thompson, 2000; Hager et al., 2012). In addition, it quickly molds or becomes ropy (Gutierrez et al., 2011). In order to expand the range of gluten-free bread with the improved consumer properties and high nutritional value, the enriching supplements from non-traditional vegetable raw materials, which contain significant quantities of nutritionally valuable substances – vitamins, fiber, protein, macro- and microelements – as well as preservative and antimicrobial compounds were used.

The possibility of using the rowan powder of the Russian origin as an enriching and preservative additive in the formulation of gluten free bread was studied.

The use of rowan powder in the gluten-free mix is proved by its rich chemical composition, high acidity (50–55 deg) and a high content of volatile acids (of 2.18 and 2.75%), which significantly affects the increase in the nutritional value and the improvement of consumer qualities, the formation of a more pronounced gluten-free bread odor and flavor. The presence of sorbic, benzoic and acetic acids possessing preservative properties will help prolong the shelf life and inhibit the development of microbial spoilage. Furthermore, the use of rowan powder will result in improved processing characteristics due to the high water absorption ability (205%) and high degree of esterification (55–78%) (Dubrovskaya et al., 2013).

The analysis of vitamin composition of the rowan powder showed high contents of ascorbic acid (40–70 g $100g^{-1}$, which technology is used as bread improver that provides improved structural-mechanical properties and improves ability of gas retention of the dough, which will lead to the increase in the specific bread volume and structure of porosity. In rowan powder vitamins – A (9–14 mg 100 $g^{-1}$), B1 (28–29 g 100 $g^{-1}$), B2 (374–431 g 100 $g^{-1}$), E (9–9.2 g 100 $g^{-1}$) and minerals - potassium (780–980 g 100 $g^{-1}$), calcium (260–300 g 100 $g^{-1}$), magnesium (78–100 g 100 $g^{-1}$), iron (8–2.4 g 100 $g^{-1}$) etc. were discovered. Their presence will provide the opportunity to increase the content of macro- and micronutrients that are available in gluten-free bread. So rowan powder is a valuable biological raw material that can be used to improve processing characteristics and gluten free bread nutritional value. To identify the optimal doses of rowan powder in the mixture, the gluten-free bread was baked. The results indicate that with the increasing dosages of powder from both the fruits of ordinary rowan and pomace of varietal rowan compressibility indices, specific volume and acidity of bread were improved (Figs 1 and 2). A significant influence of rowan powder in almost all sensory characteristics, particularly flavor, odor and color of the crumb was also observed.

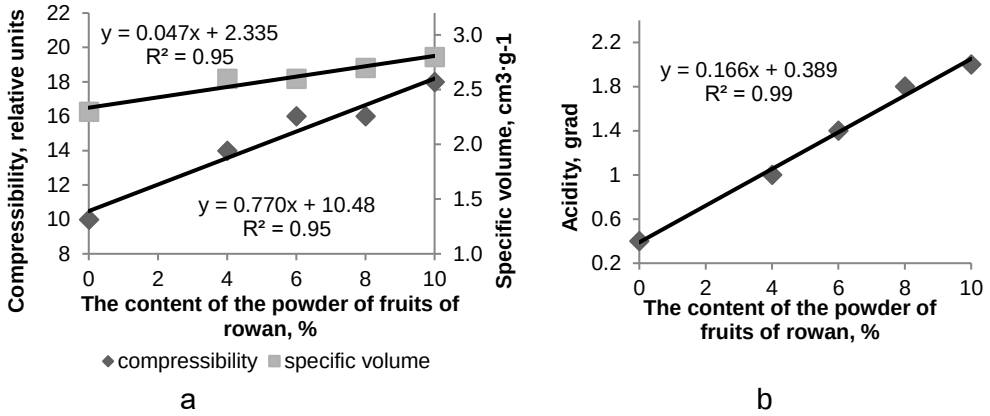



**Figure 1.** Influence of the rowan fruits powder on the compressibility, specific volume (a) and acidity (b) of gluten-free bread.

The crumb of the bread containing 4% rowan powder had a fine texture, porosity, and the odor and flavor was more pronounced and had fruit notes. It was found that the flavor depended on the type of rowan powder and its dosage. The powder of fruits of rowan, in contrast to the powder from the pomace of the rowan, had a bitterness, which manifests itself in the bread, and its intensity depends on the amount of rowan powder contained in the recipe gluten free mix. At the same time bread with powder from the pomace of rowan has gained a more pronounced fruity flavor and a harmonious acidity with the powder dosage increase (Fig. 3).

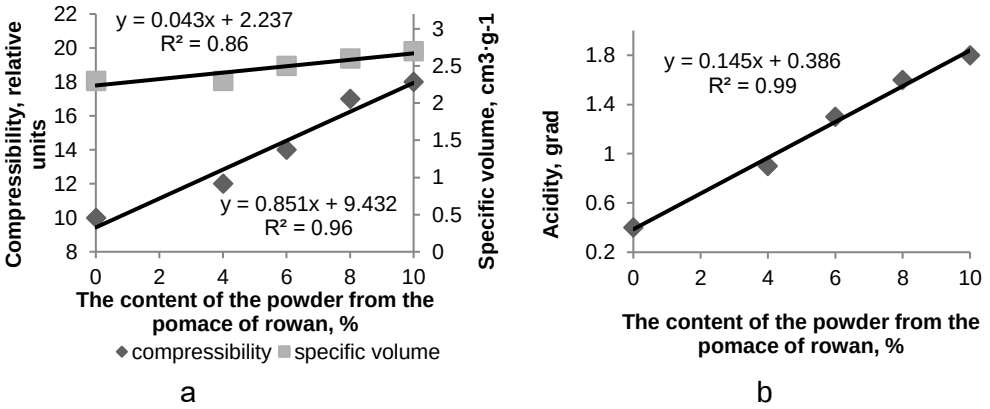



**Figure 2.** Influence of rowan fruits powder on the compressibility, specific volume (a) and acidity (b) of gluten-free bread.

It was established that the optimum amount of rowan powder in the mixture was not more than 4% of rowan fruits powder and 8% of powder from the pomace of rowan. The samples of bread were distinguished by superior organoleptic characteristics: the color of the crusts had a bright coloring, and the flavor and odor was more pronounced.

The flavor was more intense, with acidity notes, resembling traditional bread. The odor was more intense, pleasant, with fruity notes. The increase in specific volume, acidity and compressibility were observed.

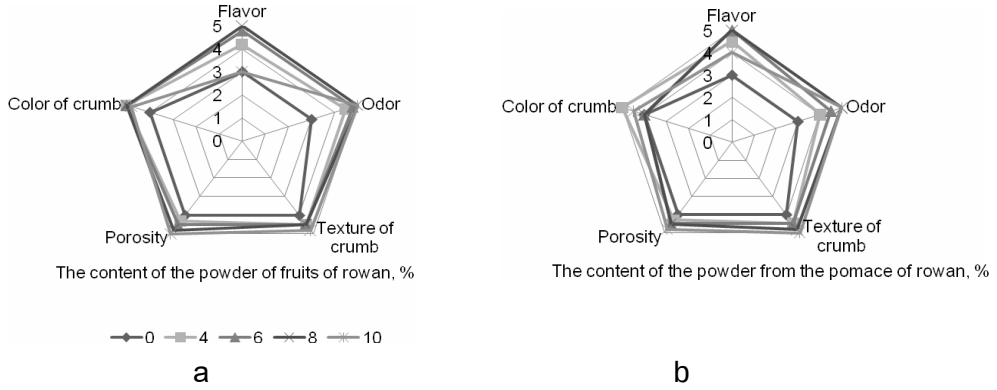



**Figure 3.** Influence of the rowan powder on the organoleptic profiles of gluten-free bread: a) rowan fruits powder; b) powder from the pomace of rowan.

To improve consumer properties of gluten free bread the research on the selection of new types of thickeners of vegetable origin of citrus fiber and pectin was conducted. Citrus fiber 'Citri-Fi 100', 'Citri-Fi 200', 'Herbacel AQ Plus', obtained by drying the cell walls of citrus fruits, water extraction or mechanical treatment without the use of chemical reagents, and citrus pectin 'Genu pectin type BIG' – highly purified pectin from citrus pomace were investigated. High water-binding capacity (from 700 to 980%), emulsifying, stabilizing and structure-forming properties allow to apply them as a consistency stabilizer to bind water and give a certain structure to the product, prolong freshness, improve appearance, crumb structure and flavor and increase nutritional value. The quality parameters of used thickeners are presented in Table 1.

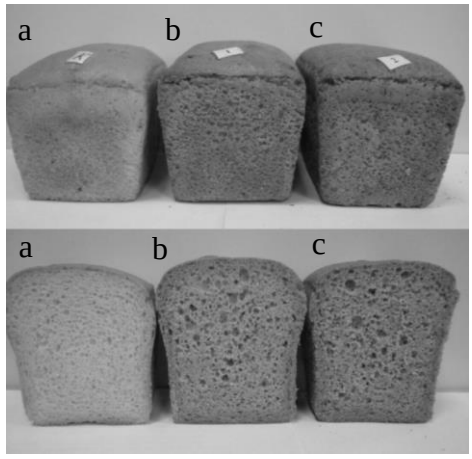



**Figure 4.** Images for gluten free bread: a) control without rowan; b) with 4% of rowan fruits powder; c) with 8% of powder from the pomace of rowan.

The parameter of water retention capacity of citrus fibres and pectin was in 2–2.6 times higher than this parameter for corn extrusion starch. Thus, it is possible to assume that the replacement of corn extrusion starch with citrus fiber and pectin will improve the structure of the dough, physico-chemical (specific volume, compressibility) and organoleptic indicators of gluten free bread quality. The use of these thickeners in the formulation of gluten free bread will increase dough water retention capacity, which will have a positive impact on the formation of crumb structure and on increasing of bread consumer advantages.

**Table 1.** Organoleptic and physico-chemical characteristics of thickeners (Mean ± SD)

| Quality parameters of used thickeners | Extrusion cornstarch | Citrus fibers | | |
|---|---|---|---|---|
| | | Citri-Fi 100 | Citri-Fi 200 | Herbacel AQ Plus |
| Mass proportion of moisture, % | 9.8 ± 0.5 | 9.6 ± 0.5 | 9.2 ± 0.5 | 9.0 ± 0.5 |
| Water-binding capacity, % | 370 ± 2.0 | 705 ± 4.0 | 710 ± 4.0 | 978 ± 5.0 |
| Content of dietary fiber, % | - | 62 ± 0.5 | 75 ± 0.3 | 90 ± 0.3 |
| Particle size, microns | 98 ± 2.0 | 200 ± 4.0 | 200 ± 4.0 | 250 ± 4.5 |
| Recommended dosage, % (by weight of the mixture) | 9.5–10.0 | 0.1–3.0 | | 0.3–1.5 |

On the basis of conducted experiments it was determined optimal dosage of thickeners in the formulation of gluten free bread, which was 1.5% – to 'Citri-Fi 100' and 'Citri-Fi 200'; 1.2% – 'Herbacel AQ Plus' and 1.3% for – 'Gene pectin type BIG' (Dubrovskaya, 2014).

In the second stage of research identified the degree of satisfaction of the average daily needs of an adult for nutrients when consuming 100 g of gluten free bread with rowan powder in accordance with the norms of physiological needs for energy and nutrients for different population groups of the Russian Federation (Tutelian, 2008).

It was found that rowan powder in combination with citrus fiber had a significant impact on the increase in the content of dietary fiber and iron (Table 2). It is important because of iron deficiency in the gluten-free diet (Thompson, 2000). The content of dietary fiber with 4% of rowan powder is 3.6 g 100 $g^{-1}$ and when using 8% pomace of rowan is 4.3 g 100 $g^{-1}$ which corresponds to the satisfaction of the daily needs for them respectively by 10.0% and 21.5%.

The iron content in the test samples compared with the control increased 2–3.5 fold, and the satisfaction of daily requirement reached 11%. Bread with 8% ash powder was characterized by a marked increase in the content of mineral elements – calcium, manganese, and potassium that corresponds, respectively, 2.4%, 14% and 4.6% of daily value.

The main sources of bio antioxidants for humans are known to be food products based on vegetable raw materials. The ash powder also contains antioxidant sources, such as vitamins C and E, selenium and carotenoids, etc. in its composition.

Consequently, the use of rowan powder in the recipe gluten free bread leads to the higher antioxidant activity, which positively influences the human health.

When determining the total content of water-soluble antioxidants on the device 'Color Jauza – 01 – AA', it was found that bread with rowanberry powder had 66.7% more water-soluble antioxidants.

**Table 2.** Satisfaction of daily requirement for nutrients

| Substance | Consumption norm (Tutelian, 2008) | Value of the indicators of satisfaction of daily requirement for nutrients in the rice gluten-free bread, % | | |
|---|---|---|---|---|
| | | Control | With rowan powder, | |
| | | | From the fruits, 4.0 % in mixture | From the pomace, 8.0 % in mixture |
| Proteins, g $day^{-1}$ | 58–117 | 5.6–11.4 | 5.7–11.5 | 5.8–11.7 |
| Fats, g $day^{-1}$ | 60–154 | 1.4–3.7 | 1.4–3.7 | 1.4–3.7 |
| Digestible carbohydrates, g $day^{-1}$ | 257–586 | 8.2–18.7 | 8.0–18.2 | 7.5–17.2 |
| Dietary fiber, g $day^{-1}$ | 20 | 4.0 | 10.0 | 21.5 |
| Vitamins: | | | | |
| C, mg $day^{-1}$ | 90 | - | 0.7 | 0.8 |
| A, mg $day^{-1}$ | 900 | - | 0.2 | 0.4 |
| E, mg/day | 15 | - | 1.4 | 2.5 |
| $B_1$, mg $day^{-1}$ | 1.5 | 0.9 | 0.8 | 0.8 |
| $B_2$, mg $day^{-1}$ | 1.8 | 0.7 | 1.1 | 1.7 |
| PP, mg $day^{-1}$ | 20 | 1.6 | 1.6 | 1.6 |
| Minerals: | | | | |
| Sodium, mg $day^{-1}$ | 1,300 | 15.2 | 15.3 | 15.5 |
| Magnesium mg $day^{-1}$ | 400 | 1.2 | 1.6 | 2.3 |
| Potassium, mg $day^{-1}$ | 2,500 | 3.2 | 4.2 | 4.6 |
| Calcium, mg $day^{-1}$ | 1,000 | 1.1 | 1.7 | 2.4 |
| Phosphorus, mg $day^{-1}$ | 800 | 3.5 | 4.6 | 5.7 |
| Iron, mg $day^{-1}$ | 10–18 | 1.7–3.0 | 6.1–11.0 | 3.3–6.0 |
| Selenium, μg $day^{-1}$ | 55–70 | - | 0.06–0.07 | 0.1–0.2 |
| Manganese, mg $day^{-1}$ | 2 | - | 3.3 | 14.0 |
| Zinc, mg $day^{-1}$ | 12 | - | 0.2 | 0.4 |

Due to the fact that rowan powder contains organic acids, which have preservative action, the effect of rowan powder on increasing of bread microbiological stability during storage was studied. The contamination of raw materials by spore-forming bacteria of the genus *Bacillus* is known to be the main cause of ropy disease.

The degree of bacteriological contamination of the main gluten-free raw material (soy protein, rice flour and corn starch extrusion and corn, Rowan powder) and the effect of the rowan powder on the quality of gluten free bread and its resistance to the ropy disease development were investigated.

The highest number of spore-forming bacteria was detected in the corn starch, rice flour and rowan powder (Table 3). Four strains of spore forming bacteria were isolated from gluten-free raw materials. It is known that a large number of bacteria can form spores, but not all of them are the causative agents of bread ropy disease. Regarding this, laboratory baking with bread crumbs, infected with spores of these bacteria was conducted to identify the ability of the isolated spore-forming bacteria that cause gluten-free bread ropy disease. A control bread sample was made from gluten free raw materials without spore infected crumbs.

**Table 3.** Microflora of gluten-free raw materials (Mean ± SD)

| Raw materials | Spore-forming bacteria x $10^{-2}$, CFU $g^{-1}$ |
|---|---|
| Corn flour | 8.0 ± 0.4 |
| Corn starch | 13.0 ± 0.6 |
| Rice flour | 12.0 ± 0.6 |
| Soya protein | 2.0 ± 0.1 |
| Starch extrusion | 2.0 ± 0.1 |
| Rowan powder | 10.0± 0.5 |

It was found that all four test samples prepared with the crumb containing strains of spore-forming bacteria isolated from raw material fell ill after 18–24 h, while in the control sample signs of the ropy disease appeared in 48 hours. It confirms the ability of the studied spore-forming bacteria to cause ropy disease of gluten free bread. Thus, the experiment has shown that gluten-free raw materials are the primary source of ropy disease.

In order to establish the effect of rowan powder on the ropy disease of bread the laboratory baking with rowan powder and breadcrumbs infected by spores were carried out. Bread was laid deposited in the precipitating conditions at 37 °C and humidity 70 ± 5%.

It was found (Table 4) that in the samples with the rowan powder in the amount of 4% signs of ropy disease appeared 12 hours later than in the controls. In the samples of gluten free bread with 8% rowan powder and infected crumbs signs of ropy disease were noted 6 hours later than in controls ones with crumbs, and in samples prepared without spore crumbs the signs of the disease were missing for 48 hours.

**Table 4.** Influence of rowan powder on the ropy disease of gluten free bread

| Indicator | Storage periods (day) to microbial spoilage in bread when making rowan powder in the amount,% | | | | | |
|---|---|---|---|---|---|---|
| | 0 | | 4 | | 8 | |
| | Without spore crumbs | 1% of spore crumbs | Without spore crumbs | 1% of spore crumbs | Without spore crumbs | 1% of spore crumbs |
| Acidity, degrees | 0.4 | - | 1.2 | - | 1.4 | - |
| Weak odor | 24 | 18 | 36 | 24 | nd | 24 |
| Strong odor | 36 | 24 | - | - | nd | - |
| The stickiness of crumb | 36 | 24 | 48 | 36 | nd | 36 |

nd – not detected for 48 hours

It was also found that the use of rowan powder helped to slow down the development of colonies of mold on bread surfaces for 12 hours.

Safety of bread with rowan powder for nutrition of people suffering from coeliac was evaluated by the content of gluten in bread by enzyme-linked immunosorbent assay (Akobeng & Thomas, 2008). The content of immunoreactive gluten was less than 10 mg per 1 kg of bread that meets the requirements of diet therapy in coeliac disease (20 mg 1 $kg^{-1}$).

Thus, it was proved that the use of rowan fruit powder and citrus dietary fiber would expand the assortment of gluten free bread, improve its physico-chemical and

organoleptic characteristics and increase the nutritional value and microbiological safety.

## CONCLUSIONS

The optimum amount of rowan powder in the gluten-free bread recipe, which is not more than 4% of rowan fruits powder and 8% of powder from the pomace of rowan, was established. It was proved that the use of rowan powder improved the organoleptic characteristics of gluten free bread and increases the acidity, specific volume and compressibility. The flavor and odor was more intense, pleasant, with acidity and fruity notes due to rowan powder in comparison to gluten free control bread.

It was experimentally established that citrus fiber and pectin improved the specific volume of the bread, the compressibility of the crumb compared to the control sample.

The content of vitamins A, E, PP, C, group b, mineral substances – iron, magnesium, calcium, potassium, selenium, organic acids, including providing the preservative effects (citric, lactic, sorbic, benzoic) was established in the rowan powder. It was established that rowan powder and citrus fiber had a significant impact on the increase in the content of dietary fiber and iron. The content of dietary fiber in bread with 4% of powder of fruits of rowan is 3.6 g 100 $g^{-1}$ and in bread with 8% pomace of rowan it is 4.3 g $100g^{-1}$ which corresponds to the satisfaction of the daily needs for dietary fiber respectively by 10.0% and 21.5%.

It was found that bread with rowanberry powder had 66.7% more water-soluble antioxidants.

The contamination of the main gluten-free raw material (soy protein, rice flour and corn starch extrusion and corn, rowan powder) and its influence on ropy disease of gluten free bread were established. Four strains of spore forming bacteria were isolated from gluten-free raw materials and their ability to cause ropy disease of gluten-free bread was proved. It was found that the use of the powder of rowan slowed down ropy disease and molds due to organic acids in its composition and the increase in acidity of bread.

## REFERENCES


Afanasjeva, O. 2003. *Microbiology of baking*. St. Petersburg, 220 pp. (in Russian).

Akobeng, A.K. & Thomas, A.G. 2008. Systematic review: tolerable amount of gluten for people with coeliac disease. *Alimentary Pharmacology & Therapeutics* **27**(11), 1044–1052.

do Nascimento, A.B., Fiates, G.M.R., dos Anjos, A. & Teixeira, E. 2013. Analysis of ingredient lists of commercially available, gluten-free and gluten-containing food products using the text mining technique. *International Journal of Food Sciences and Nutrition* **64**(2), 217–222.

Dubrovskaya, N. 2014. The use of citrus dietary fiber in the formulation of gluten free bread. *Proceedings of the II International correspondence scientific-practical conference* 95–99. (in Russian).

Dubrovskaya, N., Kuznetsova, L. & Parachina, O. 2013. Formulation of gluten-free bakery products enriched with rowanberry, *III International scientific-practical conference 'Innovative food technologies in the field of storage and processing agricultural raw materials'* 87–91. (in Russian).

Gutierrez, L., Batlle, R., Andujar, S., Sanchez, C. & Nerin, C., 2011. Evaluation of antimicrobial active packaging to increase shelf life of gluten-free sliced bread. *Packaging Technology & Science* **24**(8), 485–494.

Hager, A., Wolter, A., Czerny, M., Bez, J., Zannini, E. & Arendt E. 2012. Investigation of product quality, sensory profile and ultrastructure of breads made from a range of commercial gluten-free flours compared to their wheat counterparts. *European Food Research and Technology* **235**(2), 333–344.

Javaria, S., Marwat, S.K., Raza, S., Hameed, A. & Waseem, K. 2016. Formulation of gluten-free baked products for coeliac patients: a review of contemporary methodologies and quality improving factors. *American-Eurasian Journal Agricultural & Environmental Sciences* **16**(4), 826–835.

Krasilnikov, B. Mehdiyev, M. Domoroshchenkova, T., Demyanenko, O. & Parahina, O. 2013. The use of blue lupine in the production of gluten-free muffins. *Confectioner*y **2**, 12–17. (in Russian).

Kuznetsova, L. & Dubrovskaya, N. 2014. Polycomponent mixture for the production of gluten-free products. *Bakery production* **10**, 20–22 (in Russian).

Matos, M. & Rosell, C. 2014. Understanding gluten-free dough for reaching breads with physical quality and nutritional balance. *Journal of the Science of Food and Agriculture* **95**(4), 653–66.

Nachay, K. 2013. Putting better baked goods on the table. *Food Technology* **67**(12), 26–42.

Pellegrini, N. & Agostoni, C. 2015. Nutritional aspects of gluten-free products. *Journal of the Science of Food and Agriculture* **95**(12), 2380–2385.

Pszczola, D. 2012. The rise of gluten-free. *Food Technology* **66**(12), 55–66.

Rashtak, S., & Murray, J.A. 2012. Review article: coeliac disease, new approaches to therapy. *Alimentary Pharmacology & Therapeutics* **35**(7), 768–781.

Thompson, T. 2000. Folate, iron, and dietary fiber contents of the gluten-free diet. *Journal of the American Dietetic Association* **100**(11), 1389–1396.

Troncone, R. & Jabri, B. 2011. Coeliac disease and gluten sensitivity. *Journal of Internal Medicine* **269**(6), 582–590.

Tutelian, V.A. 2008. Methodical recommendations 2.3.1.2432 -08. 'Norms of physiological needs for energy and nutrients for different population groups of the Russian Federation'.

Vokhmyanin, N. 2009. *The modern concept of celiac disease*. St. Petersburg, 150 pp. (in Russian).

*Agronomy Research* **15**(S2), 1287–1294, 2017

# Total phenolic content and antioxidant activity of tritordeum wheat and barley

M. Eliášová* and L. Paznocht

Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Chemistry, Kamýcka 129, CZ16521, Prague 6, Czech Republic
*Correspondence: eliasovam@af.czu.cz

**Abstract.** Whole grains are a source of numerous antioxidant compounds such as phenolic compounds, anthocyanins, phenolic acids, proanthocyanidins, lignans and others which are able to scavenge free radicals. Thus cereals seem to be very useful in preventing chronic diseases like metabolic syndrome (obesity, high blood pressure, high blood triglyceride and glucose levels), diabetes, neurodegenerative diseases, cancer and chronic inflammatory diseases. Recently, there has been an increased consumer demand for cereal based foods, especially whole cereals. Such demand provides scope for innovations of which an important one is introduction of a completely new cereal cross called tritordeum. This alternative cereal, which is presented as a good source of health beneficial compounds, was assessed in this study and compared with wheat and barley. The total phenolic content (TPC) and related total antioxidant activity (TAA) were investigated via two spectrophotometric methods using a stable 2,2-diphenyl-1-picryhydrazyl radical (DPPH) and Folin-Ciocalteau reagent respectively. Both TPC and related TAA values of tritordeum and wheat were similar but were significantly lower compared to barley. Results have also revealed a close relationship between TAA and TPC ($R^2 = 0.93$, $p < 0.05$), which might suggest that increased antioxidant activity in those grains is caused by phenolic compounds contained in them. Tritordeum seems to represent a new cereal with good prospects; nevertheless more detailed analysis of its health related compounds is required.

**Key words:** cereals, DPPH, spectrophotometry, total antioxidant activity, total phenolic content, tritordeum

## INTRODUCTION

Cereals represent a staple food group in the human diet. They are a major source of energy, essential and health promoting components (Tayyem et al., 2016). Especially whole grain products are a potential source of numerous antioxidant compounds, which can be found in bran or germ of kernels (Abozed et al., 2014; Laddomada et al., 2015). Yet they are very heterogenic groups of compounds belonging to several classes such as phenolic compounds, anthocyanins, phenolic acids, proanthocyanidins, lignans and others (Okarter et al., 2010). Thanks to their antioxidant properties they can significantly contribute to the total antioxidant activity of cereal based foods (Liu, 2007; Shao et al., 2014). Nowadays, such foods are gaining importance while they seem to be very useful for prevention of human chronic diseases. Due to the increasing prevalence of metabolic

syndrome (obesity, high blood pressure, high blood triglyceride and glucose levels), diabetes, neurodegenerative diseases, cancer, chronic inflammatory diseases, insufficient fibre intake and only minimal physical activity, the overall demand for nutritionally beneficial products is growing. Consumers are interested in buying new sources (or in re-discovering the old ones) of such foods, which can help them to maintain good health. Since whole cereal products represent such commodities, grain breeders all over the world are seeking and breeding various cereal varieties/lineages with increased health benefits (Espín et al., 2007; Dykes and Roony, 2007; Syed Jaafar et al., 2013).

Genetic breeding led to creation of, besides many others, tritordeum, a cross between durum wheat (*Triticum durum* L.) and wild barley (*Hordeum chilense* L.). While wheat and barley have been common crops worldwide for millennia, tritordeum is a relatively new crop presented as an alternative cereal with unique properties and major health benefits (www.tritordeum.com). These are associated with content of nutritionally significant constituents, such as carotenoids and phenolic acids. Above all, tritordeum shows high content of carotenoids, especially lutein, which is estimated to be 5-6 times higher than in common wheat (Mellado-Ortega & Hornero-Méndez, 2015). Furthermore, another recent study identified the composition of phenolic acids in tritordeum, suggesting their contribution to health benefits of tritordeum (Navas-Lopez et al., 2014). Nonetheless, little is known about other nutritionally important constituents in tritordeum or its health associated characteristics.

A possible way of assessing cereals' health benefits is analysis of compounds contained in grains or potential health effects evaluation. Although these effects are difficult to determine they can be reflected in some detail by certain methods. For example measuring of total phenolic content (TPC) or total antioxidant activity (TAA) represents a simple way to evaluate potential nutritional significance. Total phenolic content quantifies phenolic compounds using Folin-Ciocalteu reagent assay, which is based on electron transfer and measures the ability of an extract to reduce the reagent (Margraf et al., 2015). Total antioxidant activity can be measured by various methods, for example common antioxidant assay using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). It is based on the significant ability of phenolic compounds to scavenge the DPPH free radical (Antolovich et al., 2002).

So far, no study quantifying phenolic compounds (TPC) in tritordeum and their antioxidant activity (TAA) has been conducted. Therefore the first aim of this study was to assess the overall amount of phenolic compounds in tritordeum by determination of TPC and to obtain the TAA value of phenolic compounds. The subsequent aim of this study was to verify the correlation between TPC and TAA as presented in some studies (Ivanišová et al., 2014; Zhao et al., 2006) and to compare these values to those measured in wheat and barley.

## MATERIALS AND METHODS

### Grain samples

This study evaluated several various cultivars and genotypes of three types of cereal grains – wheat, barley and tritordeum (Table 1). The cereals included red wheat, blue wheat, purple wheat, light-coloured barley, dark-coloured barley (*Hordeum vulgare* var. *nudimelanocrithon* L.) and tritordeum. The description of all cereal genotypes used in

this study is shown in Table 1. The cereal varieties were grown in the Agricultural Research Institute Kromeriz (Czech Republic) over the same period and were harvested in 2014. The analyses were performed using ground samples prepared according to Hosseinian et al. (2008). Briefly, the grain samples were ground in an IKA analytical mill (Janke & Kunkel Co., Stanfen, Germany) to pass through 0.5 mm screen (35 mesh) and homogenised well.

**Table 1.** Description of investigated cereal genotypes

| Cultivar | Species | Growth type | Country of origin* | Variety status | Grain colour |
|---|---|---|---|---|---|
| JB 1 | × *Tritordeum* Ascherson et Graebner | spring | ESP | released variety | yellow endosperm |
| JB 3 | × *Tritordeum* Ascherson et Graebner | spring | ESP | released variety | yellow endosperm |
| HT 439 | × *Tritordeum* Ascherson et Graebner | spring | ESP | breeding line | yellow endosperm |
| UC 66049 | *Triticum aestivum* L. | spring | USA | genetic resource | blue aleurone |
| Tschermaks B.S. | *Triticum aestivum* L. | spring | AUT | research germplasm | blue aleurone |
| KM 131-15** | *Triticum aestivum* L. | winter | CZE | breeding line | blue aleurone |
| Skorpion | *Triticum aestivum* L. | winter | CZE | released variety | blue aleurone |
| RU 687-12 | *Triticum aestivum* L. | spring | CZE | breeding line | purple pericarp |
| Konini | *Triticum aestivum* L. | spring | NZL | research germplasm | purple pericarp |
| Vanessa | *Triticum aestivum* L. | winter | CZE | released variety | red (standard) |
| PS Karkulka | *Triticum aestivum* L. | winter | SVK | released variety | purple pericarp |
| Bohemia | *Triticum aestivum* L. | winter | CZE | released variety | red (standard) |
| Hordeum nudimelanocrithon | *Hordeum vulgare* L. | spring | ETH | genetic resource | black grain |
| AF Cesar*** | *Hordeum vulgare* L. | spring | CZE | released variety | standard |
| AF Lucius*** | *Hordeum vulgare* L. | spring | CZE | released variety | standard |

* CZE Czech Republic, ESP Spain, AUS Austria, NZL New Zealand, SVK Slovakia, USA United States, AUT Austria, ETH Ethiopia
** origin of KM 131-15 is: (Skorpion × PS Karkulka) × (Citrus × Bona Dea)
*** barley varieties have naked (hulless) caryopsis

**Total phenolic content**

The TPC was evaluated according to Lachman et al. (2011) with minor modifications. Briefly, 2.5 g of ground grain were extracted with 25 ml of 0.1% HCl in methanol overnight at -20 °C. Each extract was centrifuged at 8,000 rpm for 10 min and 2 ml volume was reacted with 2.5 ml of Folin-Ciocalteu reagent with addition of 7.5 ml

of 20% sodium carbonate and was filled up with pure water to 50 ml. After 2 hours the solution was centrifuged at 8000 rpm for 2 min and absorbance at 765 nm was measured. The results were expressed as mg of gallic acid per 100 g of dry matter (mg GA 100 $g^{-1}$ DM).

**Total antioxidant activity using DPPH**

The DPPH radical cation scavenging activity of methanolic extracts was evaluated according to Lachman et al. (2012) with minor modifications. Briefly, 2.5 g of ground grain were extracted with methanol and shaken for 2 hours at room temperature. The extracts were filled up with methanol to 25 ml and stored in darkness at room temperature for one week. The extracts were then centrifuged at 8000 rpm for 2 min. The solution of DPPH was prepared in methanol in a concentration responding to absorbance of 0.600 ± 0.01 AU at 515 nm. 100 µl of extract was mixed with 1 ml of DPPH solution, incubated for 20 min and measured at 515 nm. The results were expressed as mg of Trolox equivalent antioxidant activity per 100 g of dry matter (mg TEAC 100 $g^{-1}$ DM).

**Statistical analysis**

All experimental data were analysed using Statistica software, version 12 (Dell Software). Analysis of variance (ANOVA) was performed with 5 replicates for each sample. Statistical significance was declared at $p < 0.05$.

## RESULTS AND DISCUSSION

Total phenolic content (TPC) and total antioxidant activity (TAA) were evaluated in varieties of tritordeum as well as wheat and barley. The measured values of TPC and TAA proved distinctions among cereal types (Fig. 1).

Tritordeum, the new cereal with possible major health benefits, showed the lowest values of both TPC and TAA. Their average values were 65.86 ± 2.68 mg GA 100 $g^{-1}$ DM and 16.30 ± 0.21 mg TEAC 100 $g^{-1}$ DM. Contrary to the JB 1 and JB 3 released varieties, the HT 439 breeding line reached higher values of TPC and TAA. Its higher content of health beneficial compounds therefore offers potential for subsequent breeding. Overall, evaluation of tritordeum showed just a little difference between tritordeum and wheat varieties, which gave slightly higher results of 73.68 ± 3.87 and 21.98 ± 1.23 for TPC and TAA respectively. These results indicate that the amount of phenolic compounds possessed by tritordeum is similar to that in wheat. Such finding therefore supplements the study of Navas-Lopez et al. (2014) who reported that the composition of phenolic compounds in tritordeum and wheat is alike. Nevertheless, as mentioned before, Mellado-Ortega & Hornero-Méndez (2015) stated that tritordeum has above all a significant content of carotenoids, particularly lutein, the amount of which can reach 5-6 times that of the amount in wheat. Since the extraction method used in this study reflects only the antioxidant activity of phenolic compounds, we can still assume that carotenoid content in tritordeum can contribute to overall antioxidant activity more than phenolic compounds.

Compared to these results, barley reached substantially higher values of both TPC ($p = 0.039$, $\alpha = 0.05$) and TAA ($p = 0.028$, $\alpha = 0.05$) than tritordeum and wheat. The average TPC of barley was 129.49 ± 5.81 mg GA 100 $g^{-1}$ DM and 98.86 ± 2.82 mg

TEAC 100 $g^{-1}$ DM. The TPC and TAA values were significantly different not only between barley on one side and wheat and tritordeum on the other side, but also among single barley varieties.

Moreover, the results showed a noticeable trend among TPC and TAA values, which were assessed by Fisher's linear correlation analysis to evaluate their relationship. The measurement proved a very close correlation between TPC and TAA as the correlation analysis gave $R^2 = 0.93$ ($p < 0.05$).

These results suggest that phenolic compounds in cereals directly affect antioxidant capacity. This fact supports the previous findings of Ivanišová et al. (2014) who reported significant correlation between TCP and AOA ($R^2 = 0.87$, $p < 0.05$) as well as those of Zhao et al. (2006) who determined an even closer relationship ($R^2 = 0.94$, $p < 0.01$) very similar to the one published in this study.

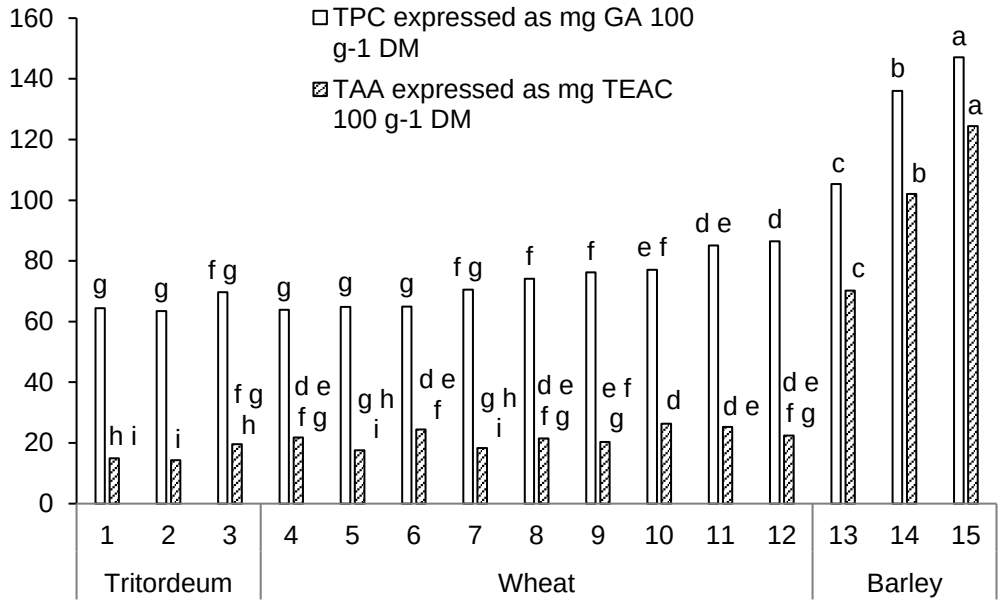



**Figure 1.** TPC and TAA values of specific cereals and their varieties. Values with the same letters are significantly different ($P < 0.05$). Different small letters indicate significant difference among samples. Tritordeum: 1 – JB 1, 2 – JB 3, 3 – HT 439. Wheat: 4 – UC 66049, 5 – Tschermak B.S., 6 – KM 131-15, 7 – Skorpion, 8 – RU 687-12, 9 – Konini, 10 – Vanessa, 11 – PS Karkulka, 12 – Bohemia. Barley: 13 – Hordeum nudimelanocrithon, 14 – AF Cesar, 15 – AF Lucius.

TPC values measured in this study are similar to results published by other researchers. Wheat varieties showed value of 73.68 ± 3.87 mg GA 100 $g^{-1}$ DM, which correspond well with Moore et al. (2005) and Lachman et al. (2011) who reported mean value of TPC in wheat 60.0 and 55.2 respectively, but are slightly lower than the values of Adom et al. (2002) and Abozed et al. (2014) who measured 136.0 and 112.0 respectively. This distinction is not substantial and might be caused by the differences between analysed varieties and the extraction methods used in these studies. The

distinction of TPC between barley and wheat, which was demonstrated in this study, was also published by Fogarasi et al. (2015).

The literature reports significant discrepancies in cereals' TAA measured using DPPH. Some researchers such as Li et al. (2007), Fogarasi et al. (2015) and Mazzoncini et al. (2015) obtained TAA mean values of wheat (as mg TEAC 100 $g^{-1}$ DM) to be 147.0, 250.0, and 2753.2 respectively. On the contrary, other authors such as Brandolini et al., Lachman et al. (2012) Yilmaz et al. (2015) reported TAA mean values of wheat as 13.8, 16.4 and 30.0 respectively. The last stated results correspond with those measured in this study. Such distinctions could be caused by diverse sample preparation or by a different method procedure. Particularly the time of incubation of the extract with DPPH can significantly affect the results – for example Fogarasi et al. (2015) let DPPH react for 60 min while Brandolini et al. (2013) for only 30 min. The shorter time of incubation quantifies rather fast reacting antioxidants while a longer time reflects the slow reacting antioxidants to a greater extent. Another significant parameter could be the presence of acid in the extracting solution. DPPH is reduced by accepting of the hydrogen atom (Mishra et al., 2012) and its decrease can be therefore negatively affected by the presence of hydrogen releasing acid. This fact might explain the distinction in values measured by Mazzoncini et al. (2015) who used acidified extract and Yilmaz et al. (2015) who used non-acidified extract.

## CONCLUSION

Tritordeum varieties showed similar total phenolic content and antioxidant activity to wheat. However, the results indicate that the breeding line HT 439 possesses slightly higher content of health beneficial compounds. More attention should be paid to this breeding line since it offers potential for further genetic breeding. Yet more analyses of tritordeum are required as this study gave just a brief overview of its characteristics.

The overall results provide evidence that cereals have significant content of antioxidants from the group of phenolic compounds, although their amounts in individual cereal types differ. The highest contents of total phenolics and related TAA were found especially in barley varieties, while values of wheat and tritordeum were significantly lower. The proven relationship between TPC and TAA suggest direct responsibility of phenolic compounds for antioxidant activity. It can be assumed that cereals are an important source of phenolics and other antioxidant compounds and that their consumption can considerably contribute to consumers' health through increase of antioxidant intake.

ACKNOWLEDGEMENTS. This work has been supported by the University Internal Grant Competition (CIGA) at the Czech University of Life Sciences in Prague, No. 20162002 and No. 20162005.

## REFERENCES

Abozed, S.S., El-kalyoubi, M., Abdelrashid, A. & Salama, M.F. 2014. Total phenolic contents and antioxidant activities of various solvent extracts from whole wheat and bran. *Annals of Agricultural Science* **59**(1), 63–67.

Adom, K.K. & Liu, R.H. 2002. Antioxidant activity of grains. *Journal of Agricultural and Food Chemistry* **50**(21), 6182–6187.

Antolovich, M., Prenzler, P.D., Patsalides, E., McDonald, S. & Robards, K. 2002. Methods for testing antioxidant activity. *Analyst* **127**(1), 183–198.

Brandolini, A., Castoldi, P., Plizzari, L. & Hidalgo, A. 2013. Phenolic acids composition, total polyphenols content and antioxidant activity of *Triticum monococcum*, *Triticum turgidum* and *Triticum aestivum*: A two-years evaluation. *Journal of Cereal Science* **58**(1), 123-131

Dykes, L. & Rooney, L.W. 2007. Phenolic compounds in cereal grains and their health benefits. *Cereal Foods World* **52**(3), 105–111.

Espín, J.C., García-Conesa, M.T. & Tomás-Barberán, F.A. 2007. Nutraceuticals: Facts and fiction. *Phytochemistry* **68**(22-24), 2986–3008.

Fogarasi, A.L., Kun, S., Tankó, G., Stefanovits-Bányai, É. & Hegyesné-Vecseri, B. 2015. A comparative assessment of antioxidant properties, total phenolic content of einkorn, wheat, barley and their malts. *Food Chemistry* **167**, 1–6.

Hosseinian, F.S., Li, W. & Beta, T. 2008. Measurement of anthocyanins and other phytochemicals in purple wheat. *Food Chemistry* **109**(4), 916–924

Ivanišová, E., Ondrejovič, M., Chmelová, D., Maliar, T., Havrlentová, M. & Rückschloss, L'. 2014. Antioxidant activity and polyphenol content in milling fractions of purple wheat. *Cereal Research Communications* **42**(4), 578–588.

Lachman, J., Miholova, D., Pivec, V., Jírů, K. & Janovská, D. 2011. Content of phenolic antioxidants and selenium in grain of einkorn (*Triticum monococcum*), emmer (*Triticum dicoccum*) and spring wheat (*Triticum aestivum*) varieties. *Plant, Soil and Environment* **57**(5), 235–243.

Lachman, J., Orsák, M., Pivec, V. & Jírů, K. 2012. Antioxidant activity of grain of einkorn (*Triticum monococcum* L.), emmer (*Triticum dicoccum* Schuebl [Schrank]) and spring wheat (*Triticum aestivum* L.) varieties. *Plant, Soil and Environment* **58**(1), 15–21.

Laddomada, B., Caretto, S. & Mita, G. 2015. Wheat Bran Phenolic Acids: Bioavailability and Stability in Whole Wheat-Based Foods. *Molecules* **20**(9), 15666–15685.

Li, W.D., Pickard, M.D. & Beta, T. 2007. Effect of thermal processing on antioxidant properties of purple wheat bran. *Food Chemistry* **104**(3), 1080–1086.

Liu, R.H. 2007. Whole grain phytochemicals and health. *Journal of Cereal science* **46**(3), 207–219.

Margraf, T., Karnopp, A.R., Rosso, N.D. & Granato, D. 2015. Comparison between Folin-Ciocalteu and prussian blue assay to estimate the total phenolic content of juices and teas using 96-well microplates. *Journal of Food Science* **80**(11), C2397–C2403.

Mazzoncini, M., Antichi, D., Silvestri, N., Ciantelli, G. & Sgherri, C. 2015. Organically vs conventionally grown winter wheat: Effects on grain yield, technological quality, and on phenolic composition and antioxidant properties of bran and refined flour. *Food Chemistry* **175**, 445–451.

Mellado-Ortega, E. & Hornero-Mendez, D. 2015. Carotenoids in cereals: an ancient resource with present and future applications. *Phytochemistry reviews* **14**(6), 873–890.

Mishra, K., Ojha, H. & Chaudhury, N.K. 2012. Estimation of antiradical properties of antioxidants using DPPH center dot assay: A critical review and results. *Food Chemistry* **130**(4), 1036–1043.

Moore, J., Hao, Z.G., Zhou, K.Q., Luther, M., Costa, J. & Yu, L.L. 2005. Carotenoid, tocopherol, phenolic acid, and antioxidant properties of Maryland-grown soft wheat. *Journal of Agricultural and Food Chemistry* **53**(17), 6649–6657.

Navas-Lopez, J.F., Ostos-Garriro, F.J., Castillo, A., Martin, A., Gimenez, M.J. & Piston, F. 2014. Phenolic content variability and its chromosome location in tritordeum. *Frontiers in Plant Science* **5**(10).

Okarter, N., Liu, Ch. S., Sorrells, M.E. & Liu, R.H. 2010. Phytochemical content and antioxidant activity of six diverse varieties of whole wheat. *Food chemistry* **119**(1), 249–257.

Shao, Y., Xu, F., Sun, X., Bao, J. & Beta, T. 2014. Identification and quantification of phenolic acids and anthocyanins as antioxidants in bran, embryo and endosperm of white, red and black rice kernels (*Oryza sativa* L.). *Journal of Cereal Science* **59**(2), 211–218.

Syed Jaafar, S.N., Baron, J., Siebenhandl-Ehn, S., Rosenau, T., Böhmdorfer, S. & Grausgruber, H. 2013. Increased anthocyanin content in purple pericarp × blue aleurone wheat crosses. *Plant Breeding* **132**(6), 546–552.

Tayyem, R.F., Bawadi, H.A., Shehadah, I., Agraib, L.M., Al-Awwad, N.J., Heath, D.D. & Bani-Hani, K.E. 2016. Consumption of whole grains, refined cereals, and legumes and its association with colorectal cancer among jordanians. *Integrative Cancer Therapies* **15**(3), 318–325.

Yilmaz, V.A., Brandolini, A. & Hidalgo, A. 2015. Phenolic acids and antioxidant activity of wild, feral and domesticated diploid wheats. *Journal of Cereal Science* **64**, 168–175.

Zhao, H.F., Dong, J.J., Lu, J., Chen, J., Li, Y., Shan, L.J., Lin, Y., Fan, W. & Gu, G.X. 2006. Effects of extraction solvent mixtures on antioxidant activity evaluation and their extraction capacity and selectivity for free phenolic compounds in barley (*Hordeum vulgare* L.). *Journal of Agricultural and Food Chemistry* **54**(19), 7277–7286.

*Agronomy Research* **15**(S2), 1295–1303, 2017

# Application of ultrasonic waves for the improvement of particle dispersion in drinks

R. Fatkullin[1,*], N. Popova[1], I. Kalinina[1] and V. Botvinnikova[2]

[1] South Ural State University, Higher Medical-Biological School, Department of Food and Biotechnologies, Lenin Ave 76, 454080 Chelyabinsk, Russia.
[2] OOVO IL Test-Puschino, 1g Gruzovaya Street, Puschino, Moscow Region 142290, Russia.
*Correspondence: fatkullinri@susu.ru

**Abstract.** Dispersion is one of the most energy-costly processes in food production. Significant proportions of hard particles remain intact when traditional dispersion methods are used. The intensification of dispersion will lead to the more effective extraction of biologically active components from raw bulk. It will also expedite the ripening of products and will improve their consumer desirability. The goal of this research was to study the dispersing effect of low-frequency ultrasound (US) on drinks which are of vegetable and animal origin (22 ± 0.6 kHz). The subjects of the research were raw cow's milk, reconstituted milk, and cranberry drinks which had been produced with the use of traditional technology and employing ultrasonic power. An ultrasonic technological device with an umbrella-shaped working element was used as an ultrasound generator (Russian Federation patent No 2141386). A Nanotrac Ultra analyser (made by Microtrac Inc, USA) was used to study particle size, using the ISO 13321 standard. An analysis of particle size was based on the method which employs the dynamic dispersion of light, in which the minimal detectable particle size is 0.8 nm. It was found that the particles in raw cow's milk, after ultrasonic processing at 180 W for dive minutes, decrease in size from 2,656 ± 72 nanometres to a prevailing particle size of 294.7 ± 24 nanometres. Following the US processing of reconstituted milk (with power at 180 W and action time at three minutes), the size of the particles decreases from 409.5 ± 62 nanometres to a prevailing particle size in the range of 202.2 ± 41 nanometres. With the cranberry drink, using ultrasound at 180 W for five minutes caused a decrease in particle size from 5,670 ± 62 nm to a prevailing size of 1,960 ± 42 nm. With an increase in ultrasound power and the duration of the application, an aggregation of particles was noted in both plant and animal-derived drinks. Therefore it can be seen that ultrasound can be used to regulate the dispersion processes in food manufacturing.

**Key words:** milk, cranberry drink, ultrasonic influence, dispersion composition

## INTRODUCTION

The dispersion of solid components in a liquid is a difficult process which requires large levels of power consumption in the manufacture of both vegetable and animal-based products. A sizable proportion of solid matter shows resistance to external influence, ie. at traditional levels of mechanical influence, a considerable share of cells cannot be destroyed (Rehbinder, 1978; Shestakov et al., 2013; Kalinina, 2015; Naumenko et al., 2016). The main technological difficulties are a result of the following

phenomenon: the low efficiency of the extraction process in the production of drinks from vegetable-based raw materials; the low efficiency levels of drink reconstitution rates from dry raw materials; a deterioration of desirable consumer properties in drinks, etc.

By intensifying the dispersion process, a number of production problems can be efficiently solved. One of the possible approaches in terms of the intensification of dispersion is by ultrasonic cavitational influence. The practicability of applying ultrasonic waves at the dispersion stage can be substantiated by the existence of a few specific intrinsic properties of ultrasonic waves.

According to the data available in current literature, the mechanism behind US acting upon colloid substances consists of the following: when ultrasound waves are sent towards a heterogeneous system, compression and rarefaction areas appear on the phases boundary, which in turn causes a rise in pressure. The overpressure formed by ultrasonic waves is added to the constant hydrostatic pressure, and this synergetic effect can reach several atmospheres. In the rarefaction phase, throughout the entire volume of liquid, in particular on the phase's boundaries in those places in which the smallest solids and gas bubbles are present, cavities are formed (Suslick, 1979; Bunkin et al., 1992; Ashokkumar et al., 2011; Shestakov et al., 2013; Krasulya et al., 2014; Porova et al., 2014; Potoroko et al., 2014).

During recompression, cavitation bubbles are collapsed, and pressure rises to a count of hundreds of atmospheres. The high intensity of the formed shockwave leads to the stress destruction of solid particles. However, it is necessary to consider the fact that, during ultrasound usage in the dispersion process, not only the destruction of particles can occur, but also the coagulation of particles caused by the destruction of the particles' solvation shells during the dispersion phase. The behaviour of cavitation processes in various environments has been actively studied by several authors (Suslick, 1979; Dezhkunov, 2001; Knorr et al., 2004; Ashokkumar et al., 2008; Shestakov et al., 2013; Krasulya et al., 2014; Porova et al., 2014; Potoroko et al., 2014). As a result of their research the applicability of ultrasonic waves for food processing has been fully proven.

The French scientist, Chemat (2011), demonstrated the applicability of ultrasonic wave technology in the manufacture of natural products on the basis of eco-extraction processes. The research focused on innovative methods in terms of nutrient extraction from various vegetable-based raw materials. Dincer et al. (2016), showed the applicability of ultrasonic waves in milk processing technology, focusing on lactose crystallisation. Huang et al. (2007), Ashokkumar et al. (2008), Zuo et al. (2012), and Bai et al. (2016) describe the application of ultrasound for starch modification. Research carried out by Bykov et al. (2011) confirms the suitability of cavitation processing for the production of non-starchy polysaccharides by means of the destruction of plant cell walls. A number of research projects have also been carried out when it comes to the field of modifying the properties of raw whole milk as used for the manufacture of fermented milk products and farmer's cheese (Khmelev, 1997; Potoroko & Tsirulnichenko, 2013; Shestakov et al., 2013; Krasulya et al., 2014; Porova et al., 2014).

The goal for this research project was to study the dispersal effect of low-frequency ultrasound ($22 \pm 0.6$ kHz) on drinks of vegetable and animal origin (raw cow's milk, reconstituted milk, and cranberry drinks), using a laser dynamic light scattering method. An ultrasonic technological device with an umbrella-shaped working element was used as an ultrasound generator (Russian Federation patent No 2141386).

## MATERIALS AND METHODS

For the purposes of this research, raw milk, reconstituted milk, and cranberry berry drink were used.

The *raw milk* (produced during the summer lactation period) was delivered from a farm belonging to Agrostimul LLC, Kurgan Region, Russia (the average content of the milk included: dry fat-free substances, 8.57%; protein, 3.17%; and fat, 3.6%).

In order to produce *reconstituted milk*, powdered skimmed milk was used which was produced using a spray-drying method by Chebarkul Lactic Plant JSC, Chelyabinsk Region, Russia (the average content of the powdered milk included: water, 2.85%; protein, 2.69%; and fat, 1.1%).

Reconstituted milk produced using traditional milk reconstitution technology saw powdered milk added to water at a temperature of between 38–45 °C, at a ratio of 1:10, which was then actively mixed and maintained for three hours, with this being used as a reference sample.

Reconstituted milk which was processed using ultrasound was produced by means of applying an ultrasound treatment to a water and powdered milk mix immediately after the mixing process had been completed. In terms of the production of this sample, milk reconstitution was specified without the preliminary heating of the water.

For the *cranberry drink* which was processed using ultrasound production, frozen cultivated cranberries were used, produced by Fresh LLC, Chelyabinsk, Russia.

As a reference sample, thawed and crushed cranberries were added to water at a ratio of 1.5:10, and then the mixture was heated to a temperature of 45 °C, mixed actively (no less than thirty times per minute), and filtered through an eight layer textile filter.

The cranberry drink was processed using ultrasound, with thawed and crushed cranberries being added to water which was at room temperature (20 °C) at a ratio of 1.5:10; the mixture is processed using ultrasound and is filtered through an eight layer textile filter.

All of the samples were treated with ultrasound at the following parameters: a frequency of $22 \pm 1.65$ kHz; an intensity of 10 W/cm$^2$; and at a power of 180 W. The exposure time was one, three and five minutes; and the liquid volume was 200ml.

A Volna-M UZTA-0.4/22-OM model submersible ultrasonic device with an umbrella-shaped working element was used as an ultrasound generator for treating the substances in the study. Its technical specifications are shown in Table 1 (Khmelev, 1997).

The ultrasonic vibrating system uses annular piezoelectric elements and is made of BT5 titanium alloy. Its operating principle is based on high-intensity ultrasonic waves propagating in fluid and in fluid-dispersed substances. The engineering solutions used are protected by Russian Federation patent No 2141386 (Khmelev, 1997).

The unique feature of this ultrasonic equipment consists of the applied design behind the ultrasonic oscillatory system, namely the umbrella-shaped tool with a concentrator connector which has a connecting size that is less than the size of the radiating surface. By selecting the optimum ratio for the maximum radiating surface diameter of the umbrella-shaped working tool so that it best matches the diameter of the concentrator end face to which the tool is connected, it is possible to provide a relative reciprocating movement for the working tool's radiating surface, ie. to increase the radiation surface area with an amplitude which corresponds to the increased concentrator

amplitude, and thereby to increase energy output in proportion to the square of the diameter; and also to create flexural oscillations in the peripheral area of the working tool's surface. Such an approach allows for the creation of acoustic flows during the course of ultrasound emissions, thereby making it possible to displace the cavitation cloud which interferes with the acoustic energy output from the working tool's end face. This provides for additional agitation of the technological material being processed, and prevents the transition of titanium into the processed liquids.

**Table 1.** Technical specifications of Volna-M UZTA-0.4/22-OM device

| **Specification** | **Value** |
|---|---|
| Ultrasound oscillation frequency, kHz | 22 ± 1.65 |
| Power, W | 400 |
| Range of power adjustment, % | 30–100 |
| Intercity of ultrasound, not less than W/cm$^2$ | 10 |
| Power supply voltage, V AC | 220 ± 22 |
| Maximum continuous operation time, hours | 8 |
| Diameter of emitting surface, mm | 25 |
| Overall dimensions: | |
| electronic generator, mm | 300x280x110 |
| oscillating system, mm | Ø70x150 |
| diameter of working element, mm | 25 |

The optimum ratio for the radiating surface diameters in relation to the connecting size increases the device's efficiency by up to 80% (Lebedev et al, 2003; Khmelev et al, 2005).

A Nanotrac Ultra analyser (made by Microtrac Inc, USA) was used for particle size analysis. The measurement procedure carried out with the Nanotrac meets the requirements of ISO 13321. The device's operating principle is based on the passing of a laser beam through liquid, the beam's reflection off moving particles, and its return to the device detector. The particle size is calculated on the basis of the expansion of the reflected ray spectrum. The method for determining particle size distribution is based on an analysis of Doppler spectral shifts. The sample placed into the cell is exposed to laser radiation, and the scattered light caused by Brownian motion of particles is recorded at an angle of 180°. The minimum size of the particles which can be found by the device is 0.8 nanometres; the measurement data are highly precise and can easily be reproduced (Dalgleish, 1995; Alexander & Dalgleish, 2006; Nobbmann et al., 2007).

A typical example of particle distribution curves in terms of dispersal systems for the drinks samples are presented in Fig. 1. All particle distribution curves for dispersal systems for the drinks samples possess a similar overall character, but they differed in terms of the value and location of peaks which correspond to different fractions of particles.

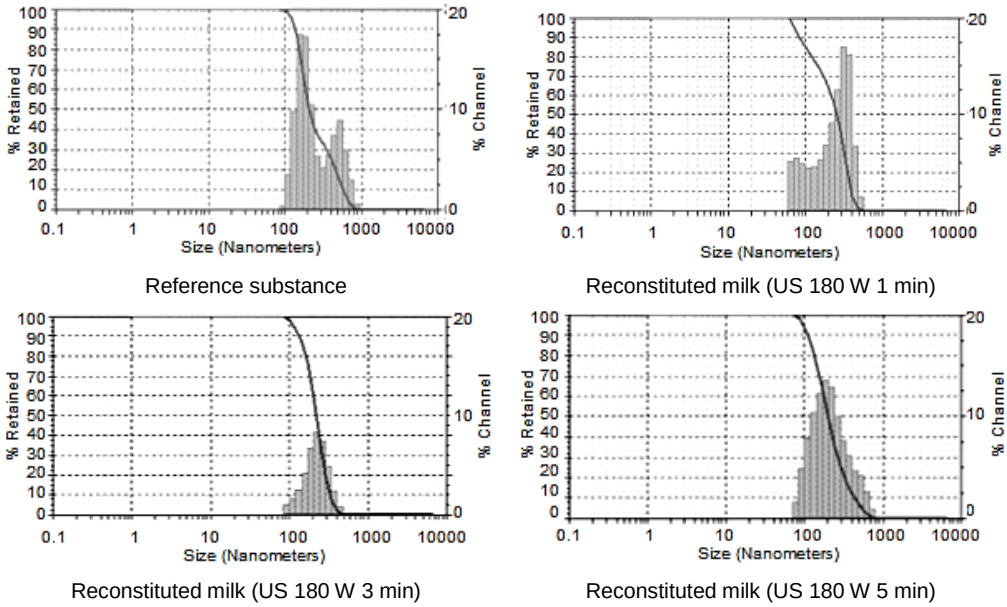



**Figure 1.** Plots for particle dispersion systems for milk reconstituted with water.

## RESULTS AND DISCUSSION

It is well known that milk is a composite polydisperse system in which dispersion phases exist in various states. In particular, protein is present in the form of colloid particles, and the size of these particles can vary from fifteen to 300 nanometres; fat is present in the form of coarse-dispersion particles of various sizes (the diameter of milk fat globules is between 0.1 and 20 microns). Other milk components exist in an ion-molecular state with particle sizes of about one nanometre or less. There is no definite difference between phases, as the aqueous solution of one substance can be a dispersion medium for others (Potoroko & Kalinina, 2010; Hussein et al, 2011; De Kruif & Huppertz, 2012; Tepel et al, 2012). When a close interrelation between phases is reached, a uniformly balanced milk system is formed. Milk dispersion has important technological value for the manufacture of dairy products as it provides the best bioavailability and digestibility of the main components of milk (Potoroko & Kalinina, 2010; Tepel et al, 2012).

The results of dispersion degree analysis for milk systems, for various US processing modes, are given in Fig. 2.

The above results for dispersible analysis show that milk is very sensitive to the ultrasonic cavitation effect, which causes an increase in droplet sizes when the droplet appears in the rarefaction area, and subsequent division into smaller droplets. Ultrasonic cavitation causes changes in the product dispersion state. It can be seen from the results that the distribution of the samples' fractional composition changes; however, different ultrasound modes influence the product system differently. An increase in ultrasonic exposure causes particle sizes to be more equal. In the reference sample for milk (whole milk which was not processed), particles of between 1,000 and 3,000 nanometres were

discovered with a particle dominance of 2,656 ± 72 nanometres. For samples exposed to US at the power of 180W for five minutes, the fractional composition changed: particles of two fractions are present: 1,461 ± 30 nanometres at 8.4%; and 294.7 ± 24 nanometres at 91.6%.

The results demonstrate the clear influence of the cavitation process on milk particle size, with the primary focus being on the size of milk fat globules.

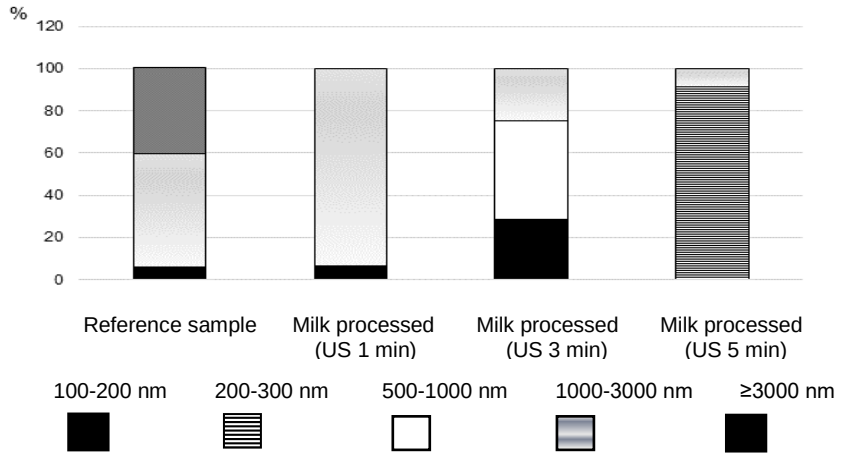



**Figure 2.** Proportions of size fractions in samples of whole milk, %.

In powdered milk production, it is necessary to strictly control the production technology in order to ensure a balance between separate components in the system, and to ensure the product's digestibility. One way of attaining natural-product properties for reconstituted milk is to achieve the required dispersion state for the system components. Fig. 3 shows the particle size for the reconstituted reference milk sample and the sample with ultrasonic treatment, determined for different time exposures.

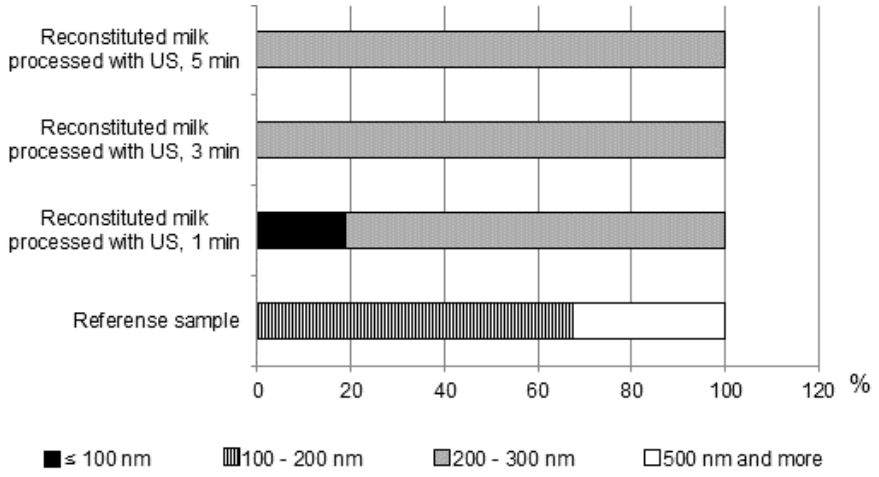



**Figure 3.** Proportions of size fractions in samples of reconstituted milk, %.

As can be seen from the dispersion analysis data, the structure of reconstituted milk is a composite polydisperse system which is characterised by the presence of various size particles. The reference sample is characterised by the availability of two size fractions: about 41% of the particles have an average size of 409 ± 62 nanometres, and 53% have an average size of 174 ± 18 nanometres; the difference between these values defines the heterogeneity of the system.

Due to the emergence of cavitational processes and additional energy influencing particles of powdered milk, ultrasound exposure facilitates the formation of a uniform milk structure with a particle fraction strictly within 200.2 and 305.6 nanometres, with a prevailing particles size of 202.2 ± 41 nanometres.

It was discovered that ultrasound exposure for one minute reduces the average phase particle sizes by 42.3% and 53.1%, and ultrasound exposure for three and five minutes balances the milk structure to particles of a single size range. At the same time, if the UZV duration is increased (to three and five minutes), the tendency of slight particles to agglomerate in reconstituted milk can be observed, but the 100 nanometres particles fraction had disappeared, even though it had been found in those samples which had been exposed to US for one minute.

Also, it should be noted that the reference sample was reconstituted using a traditional technique, with water preliminarily heated to 38–45 °C. For sample production using ultrasound exposure, the water is not heated, ie. reconstitution occurs only due to mechanical and cavitational effects. Omitting additional heating eliminates the risk of there being any loss of the natural properties (coagulation) in terms of serum proteins, and also the risk of there being a decrease in vitamin content; and besides this, the process makes it possible to intensify technological processes for the manufacture of reconstituted milk products with a decrease in cost, because there is no need to heat the mix.

For the cranberry drink which was produced using ultrasonic treatment, the size range of disperse particles also changes significantly. Particles with a size of 5,670 ± 62 nanometres were detected mainly in reference samples from the extract. For the cranberry drink sample which had been treated with US (for one minute), particles were observed within the range of 743 to 5,200 nanometres, with a dominance of particles of 5,200 ± 41 nanometres (45.6%), and 1,960 ± 42 nanometres (33.1%). An increase in the duration of ultrasonic treatment to five minutes caused the emergence of particles of a size of 236 ± 10 nanometres (11.3%). At the same time, with ultrasonic processing at 180W running for five minutes, agglomeration took place in the cranberry extract and particles were formed of a size of 5,830 ± 30 nanometres.

## CONCLUSIONS

The results obtained demonstrate the significant influence of cavitation processes on the dispersion composition of drinks both of vegetable and animal origin.

The dispersion analysis results show that milk, both natural and reconstituted, is very sensitive to ultrasonic cavitation. The fractional composition of cranberries also changed under the influence of ultrasound, enabling a decrease in particle size by 24 times. At the same time, the ability of ultrasound was observed, in some modes, to cause the agglomeration of dispersible particles in the drinks samples. This result shows

that ultrasonic cavitation makes possible a reduction in the thermal load of milk's raw materials which will preserve the natural components, providing an alternative to the homogenisation process.

In the production of cranberry drinks, ultrasound is able to intensify the extraction processes via the additional refinement of plant cells, and to improve the consumer appeal of the drinks, which are also positive factors when it comes to manufacturing technology in general.

ACKNOWLEDGEMENTS. The work was supported by Resolution 211 of the Government of the Russian Federation and Agreement № 02.A03.21.0011. Studies were carried out with financial support from the Assistance Fund of the UMNIK Programme. This article was written with support from the Government of the RF (Resolution №211 of 16.03.2013), Agreement № 02.A03.21.0011, and subsidies for the fulfilment of a fundamental part of a state order under Project № 40.8095.2017/BCh.

## REFERENCES

Ashokkumar, M., Sunartio, D., Kentish, S.E., Mawson, R., Simons, L., Vilkhu, K. & Versteeg, C. 2008. Modification of food ingredients by ultrasound to improve functionality, Innov. Food Sci. Emerg. Technol. **9**, 155–160.

Ashokkumar, M., Rink, R. & Shestakov, S. 2011. Hydrodynamic Cavitation – an Alternative to Ultrasonic Food Processing. *Electronic Journal* '*Technical Acoustics*' **9**. Available at: http://www.ejta.org.

Alexander, M. & Dalgleish, D.G. 2006. Dynamic light scattering techniques and their applications in food science. *Food Biophys*. **1**, 2–13.

Bai, W., Hébraud, P., Ashokkumar, M. & Hemar, Y. 2016. Investigation on the pitting of potato starch granules during high frequency ultrasound treatment, *Ultrason. Sonochem*. **35**, 547–555.

Bunkin, N. F. & Bunkin, F. V. 1992. Babstons, stable gas microbubbles in strongly dilute solutions of electrolytes. *JETP*. **101**(2), 512–527.

Bykov, A.V., Mezhueva, L.V., Miroshnikov, S.A., Bykova, L.A. & Tyshchenko, V.M. 2011. Prospects of use of cavitation hydrolysis of non-starch polysaccharides. *Vestnik of OSU*, **4**(123), 123–127. (in Russian).

Dalgleish, D.G. & Hallett, F.R. 1995. Dynamic light scattering: applications for food systems. *Food Res. Int.* **28**, 181–193.

De Kruif, C.G. & Huppertz, T. 2012. Casein Micelles: Size Distribution in Milk from Individual Cows. *J. Agric. Food Chem.* **60**, 4649−4655.

Dezhkunov, N.V. 2001. Sonoluminescence emission in interacting fields of highly different frequencies, *Technical Physics Letters* **12**, 15−22.

Dincer, T. & Zisu, B. 2016. Sonocrystallization of Lactose. *Handbook of Ultrasonics and Sonochemistry*, **1**, 1403–1434.

Chemat, F. 2011. Eco-Extraction of Vegetal: innovative processes and alternative solvents. *DUNOD*, Paris, 336 pp. (in French).

Huang, Q., Li, L. & Fu, X. 2007. Ultrasound effect on the structure and chemical reactivity of cornstarch granules, *Starch/Stärke* **59**, 371–378.

Hussein, R., Gaiani, C. & Scher, J. 2011. Revealing casein micelle dispersion under various ranges of NaCl: Evolution of particles size and structure. *Eng. Tech.* **51**, 972–982.

Kalinina, I. V. 2015. Innovative approaches in the formation of consumer properties of socially important groups of food *Bulletin of SUSU, series: Economy and management*, **3**, 180–184.

Khmelev, V.N. & Popov, O.V. 1997. Multifunctional Ultrasonic Devices and Their Application in Small Industries, Agriculture and at Home: A Monograph. *Barnaul Univ.* Altai State Technical University, 168 pp. (in Russian).

Khmelev, V.N., Tchyganok, S.N., Barsukov, R.V. & Lebedev A.N. 2005. Designing and Efficiency Analysis of Half-Wave Piezoelectric Ultrasonic Oscillatory Systems. *International Siberian Workshop and Tutorials on Electron Devices and Materials Proceeding EDM'2005*, *Workshop Proceedings.* Novosibirsk, **1**, 82–85 (in Russian).

Knorr, D., Zenker, M., Heinz, V. & Lee, D.-U., 2004. Applications and potential of ultrasonics in food processing. *Trends Food Sci. Techn*. **15**, 261–266.

Krasulya, O.N., Bogush, V.I. & Kochubey-Litvinenko, O.V. 2014. Food Sonochemistry in Dairy Technology. *Milk River*. **3**(55), 14–16 (in Russian).

Lebedev, A.N., Tsyganok, S.N., Khmelev, V.N., Savin, I.I., Steer, V.N. & Khmelev, M.V. 2003. Designing of Ultrasonic Oscillation Systems for Technological Devices. *Siberian Russian Workshops and Tutorials on Electron Devices and Materials EDM'2003. Workshop Proceedings*, Novosibirsk, **1**, 205–208 (in Russian).

Naumenko, N.V. & Kalinina, I.V. 2016. The influence of sonochemistry effects on adjustments of raw materials and finished goods properties in food production. *International Conference on Industrial Engineering* **870**, 691–696.

Nobbmann, U., Connah, M., Fish, B., Varley, P., Gee, C., Mulot, S., Chen, J., Zhou, L., Lu, Y., Sheng, F., Yi, J. & Harding, S.E. 2007. Dynamic light scattering as a relative tool for assessing the molecular integrity and stability of monoclonal antibodies. *Biotechnol.Genet. Eng. Rev.* **24**, 117–28.

Porova, N.P., Botvinnikova, V.V., Krasulya, O.N., Cherepanov, P. & Potoroko, I.Y. 2014. Effect of Ultrasonic Treatment on Heavy Metal Decontamination in Milk. *Ultrasonics Sonochemistry* **21**(6), 2107–2111.

Potoroko, I. Y. Kretova, Y.I. & Kalinina, I. V. 2014. Practical aspects of the use of resource efficient technologies in food production as a factor in the quality of finished goods. *Goods manager of food products* **10**, 8–13.

Potoroko, I.Y. & Tsirulnichenko, L.A. 2013. Improvement of the rheological characteristics of meat emulsions-based food sonochemistry. *Trade and Economic Problems of Regional Business Space* **1**, 306–309.

Rehbinder, P. 1978. Surface Phenomena in Disperse Systems. *Physico-Chemical Mechanics. Nauka*, Moscow, 371 pp. (in Russian).

Suslick, K. S. 1989. The chemical effects of ultrasound. *Scientific American*. 80–86.

Shestakov, S.D., Krasulya, O.N., Bogush, V.I. & Potoroko, I.Y. 2013. Technology and equipment for food processing environments using cavitation disintegration. *GIORD Publ*. 152.

Tepel, A. 2012. Chemistry and Physics of Milk. *Professiya,* St. Petersburg, 832 pp. (In Russian).

Zuo, Y.Y.J., Hébraud, P., Hemar, Y. & Ashokkumar, M. 2012. Quantification of high-power ultrasound induced damage on potato starch granules using light microscopy, *Ultrason. Sonochem*. **19**, 421–426.

*Agronomy Research* **15**(S2), 1304–1315, 2017

# Improvement of microbiological safety and shelf-life of pulse spreads through *sous vide* and high pressure processing

A. Kirse*, R. Galoburda, S. Muizniece-Brasava, D. Karklina and L. Skudra

Latvia University of Agriculture, Faculty of Food Technology, Department of Food Technology, 22 Rigas Street, LV3004, Jelgava, Latvia
*Correspondence: asnate.kirse@llu.lv

**Abstract.** Microbiological quality of *sous vide* treated (80 °C/15 min) and high pressure processed (700 MPa/10 min/20 °C) cowpea (*Vigna unguiculata* (L.) Walp. cv. Fradel) and maple pea (*Pisum sativum* var. *arvense* L. cv. Bruno) spreads in flexible vacuum packaging during 62-day storage at 5 ± 1 °C were assessed. Pulse spreads, made from cooked pulses with salt, citric acid, oil, and seasoning, were filled in PA/PE or PET/ALU/PA/PP flexible film pouches, packaged in vacuum (20 mbar) and hermetically sealed. Microbiological testing was performed by determining total plate count, yeast and mould count on days 0, 15, 29, 42, 50, 57, and 62, and the presence of *B. cereus*, *C. perfringens* and *E. coli* before processing and after 62-day storage. The results showed that total plate count increased significantly after 62-day storage in both *sous vide* ($p = 0.011$) and high pressure processed ($p = 0.017$) spreads; the observed over one-log increase was without significant differences between pulse spreads and packaging materials ($p < 0.05$). The admissible level of total plate count ($N_{max} < 3.69$ log CFU g$^{-1}$) in pulse spreads was not exceeded. The presence of yeasts and moulds, *C. perfringens* and *E. coli* in pulse spreads was not confirmed, whereas *B. cereus* accounted to $<10^2$ CFU g$^{-1}$ after 62-day storage. The suggested shelf-life of processed pulse spreads is 62 days; except for *sous vide* treated spreads with seasoning in both packaging materials – 57 days. Both processing methods are suitable to ensure the production of high quality pulse spreads with adequately long shelf-life.

**Key words:** cowpea, maple pea, microbiological quality, pathogens, flexible packaging

## INTRODUCTION

Pulse spreads are an innovative product made from rehydrated cooked seeds of pulses (Kirse & Karklina, 2015). They are an alternative to traditional animal-derived spreads, and have all nutritious components of pulses: high quality plant protein, complex carbohydrates, soluble and insoluble dietary fibre, a wide range of minerals and vitamins (Kirse et al., 2016a). However, shelf-life of fresh spreads is less than six days at refrigerator temperature (Kirse & Karklina, 2015). The high moisture content (65–68%), water activity (above 0.97), and the typical pH range (5.2–5.5) of the product makes pulse spreads susceptible to microbial and sensorial changes thus affecting the product shelf-life (Keenan et al., 2011; Yamani & Mehyar, 2011).

Several studies have described the application of *sous vide* treatment (Levkane et al., 2010; Baldwin, 2012; Sansone et al., 2012) and high pressure processing (Balasubramaniam, 2010; Vercammen et al., 2011; Georget et al., 2015) to increase shelf life of foodstuff by inactivating microorganisms and improving microbial safety of food

products (Yordanov & Angelova, 2010). Both methods are suitable post-processing methods for shelf-life extension of various ready to eat products. Vacuum packaging in flexible polymer pouches is a fundamental technology of *sous vide* treatment and high pressure processing (Brody et al., 2008), which is carried out to eliminate the risk of recontamination during storage, reduce aerobic bacterial growth and prevent leaking of food constituents during processing, resulting in flavourful and nutritious food with long shelf-life (Church & Parsons, 2000; Seydim et al., 2006).

With regards to *sous vide* treatment, the effect of temperature on microorganisms has been studied extensively (Smelt & Brul, 2014), concluding that thermal treatment at 70 °C for 2 min is sufficient to eliminate non-spore-forming pathogens such as *Listeria monocytogenes*, *Salmonella* and *E. coli* 0157 (Juneja & Snyder, 2007). Literature data suggests that high pressure inactivates microorganisms by interrupting cellular functions responsible for reproduction and survival (Torres & Velazquez 2008); the permeabilization of the cell membrane is reversible at low pressures (up to 200 MPa), but irreversible at higher pressures (Rastogi et al., 2007). Both processing methods, however, cannot eliminate microorganism spores, as spores are heat-tolerant at temperatures below 100 °C (Baldwin et al., 2012) and pressure-tolerant at pressures above 1,000 MPa (Zhang & Mittal, 2008). Current high pressure processed products in the market rely on refrigeration, similar to pasteurization (Ates et al., 2016).

In addition to microbial safety, undesirable sensory and physicochemical changes during storage can lead to food products of inadequate quality. Therefore, shelf-life prediction is based on several deterioration factors – microbiological, sensory and physicochemical parameters (Dermesonluoglu et al., 2016). It is important to evaluate shelf-life of innovative products before they can be commercialised. So far there is little to none research on spreads made from pulses, therefore, shelf-life studies are necessary as the growth of microorganisms can vary between food matrices. The aim of this study was to determine microbiological quality of *sous vide* treated (80 °C/15 min) and high pressure processed (700 MPa/10 min/20 °C) cowpea (*Vigna unguiculata* (L.) Walp. cv. Fradel) and maple pea (*Pisum sativum* var. *arvense* L. cv. Bruno) spreads in flexible vacuum packaging during 62-day storage at 5 ± 1 °C followed by shelf-life assessment.

## MATERIALS AND METHODS

### Materials

Two type's pulses growing in Europe were used to prepare pulse spreads: cowpeas (*Vigna unguiculata* (L.) Walp. cv. Fradel), harvested in 2013, Portugal and maple peas (*Pisum sativum* var. *arvense* L. cv. Bruno), harvested in 2014, Latvia. Additional ingredients for the preparation of spreads were canola oil (Iecavnieks Ltd., Latvia), citric acid (Spilva, Ltd. Latvia), Himalayan salt (Pakistan) and bruschetta (dried tomato, garlic and basil) seasoning (P.P.H. fleischmann schaft®-Polska Sp. z o.o., Poland).

### Preparation and packaging of pulse spreads

Each type of pulses was separately soaked in water (with $NaHCO_3$, 21.5 g $kg^{-1}$) at 20 ± 2 ºC for 15 h, then rinsed and boiled in a pressure cooker (KMZ, USSR) until tender (about 35 ± 5 min plus 15 min for natural pressure release) as described by Kirse & Karklina (2015). Still warm cooked pulses were then grinded in a food processor (Philips HR 7761/00, Philips, The Netherlands) together with additional ingredients. Four

different pulse spreads were studied: cowpea spread without and with bruschetta (1%), maple pea spread without and with bruschetta (1%). The physicochemical parameters characterising spreads were as follows: 0.38% salt, 66.9–67.7% moisture, $a_w$ = 0.977-0.978 and pH 5.38–5.49.

Transparent polyamide / polyethylene (PA/PE) film pouches (film thickness 60 ± 3 μm, PTC Ltd., Latvia) and light proof polyethylene terephthalate / aluminium / polyamide / polypropylene (PET/ALU/PA/PP) film pouches (film thickness 80 ± 3 μm, Nordvak Ltd., Latvia) with dimensions 45 × 170 mm were used to package prepared pulse spreads (50 ± 1 g). Two different packaging materials were used to determine whether the additional PET and ALU layers of the light proof film could provide a better quality for pulse spreads during storage. Filled pouches were hermetically sealed under vacuum (20 mbar, sealing time 1.9 s for PA/PE and 3.4 s for PET/ALU/PA/PP) using chamber type vacuum packaging machine (C300, Multivac Ltd., UK).

**Processing treatments and storage conditions**

Processing treatments of pulse spreads were carried out as described by Kirse et al. (2016c). *Sous vide* treatment was performed by pasteurizing samples for 15 min at 80.0 ± 0.5 °C (core temperature 78.0 ± 0.5 °C was reached within 9 min of treatment) in water bath (Clifton Food Range, Nickel-Electro Ltd., UK) followed by immediate chilling of samples in 2 ± 1 °C ice-water to 5 ± 1 °C temperature.

High pressure processing was performed by pascalising samples for 10 min at 700 MPa pressure in Iso-Lab High Pressure Pilot Food Processor (S-FL-100-250-09-W, Stansted Fluid Power Ltd., Essex, UK) in a 2.0 L pressure vessel with isopropanol, water mix (1:2) as the pressure transmitting liquid. High pressure processing was carried out at an ambient temperature (20.0 ± 1.0 °C), however, during processing temperature rise was observed due to the pressure increase in the vessel and reached a maximum of 40 °C; the pressure drop lowered the temperature to 10 °C. Processing regimes were chosen according to the previous investigations where *sous vide* treatment for 15 min at 80 °C (Kirse et al., 2016b) and high pressure processing for 10 min at 700 MPa (Kirse et al., 2015) showed the highest potential for shelf-life study based on lower microbial count and resource efficiency.

Untreated and processed samples were stored in a commercial display cooler (Snaige Ltd., Lithuania) with tempered glass door under daylight luminescence with radiant fix at 400 to 1000 lx (measured by LX-107 Portable Digital Light meter, Lutron Electronic Enterprise Co., Ltd., Taiwan) for 62 days at 5 ± 1 °C temperature.

**Microbiological analysis**

Microbiological testing of pulse spreads during storage was carried out according to preparation of test samples, initial suspension and decimal dilutions for microbiological examination (ISO 6887-4:2003). Peptone water (0.1% w $v^{-1}$) tenfold dilution aliquots were obtained after homogenization in a stomacher (BagMixer400, Interscience, USA) for 10 seconds. Triplicate plates were prepared using pour plate method for enumeration of total plate count (aerobic and facultative anaerobic, mesophilic bacteria, hereafter referred to as TPC) on Plate Count Agar (Ref. 01-161, Scharlau, Spain, incubation at 30 °C for 72 h) and yeasts and moulds on Malt extract agar (Ref. 01-111, Scharlau, Spain incubation at 27 °C for 48 h). Standard methods were used to determine the presence of such pathogens as *Bacillus cereus* (ISO 7932:2004),

*Clostridium perfringens* (ISO 7937:2004) and *Escherichia coli* (ISO 7251:2005). After incubation, the colonies were counted using automated colony counter aCOLyte (Topac Inc., USA) and reported as colony forming units (CFU).

Microbiological analysis of total plate count, and yeast and mould count were carried out during storage at days 0, 15, 29, 42, 50, 57, and 62. Pathogens were determined in pulse spreads before processing and after 62-day storage. Data are expressed as log colony forming units per gram of product (log CFU $g^{-1}$).

**Statistical analysis**

The processing of obtained data was performed using mathematical and statistical methods with 'Microsoft Office Excel v16.0' (Microsoft Corp., Redmond, WA); differences among results were analysed using one-way analysis of variance. Each sample was analysed in triplicate and results were given as mean ± standard deviation. Differences were considered significant at $p \leq 0.05$.

## RESULTS AND DISCUSSION

Microbiological parameters that determine the quality of pulse spreads are total plate count (TPC), count of yeasts and moulds, presence and count of pathogens (*E. coli, B. cereus, C. perfringens*), which have been defined by the European Commission (EC Regulation No 2073/2005) and the Cabinet of Ministers of the Republic of Latvia (Regulation No 461/2014). CM Regulation No 461/2014 on microbiological criteria for vegetable jams, purées and similar products suggest total plate count of such products below $5 \times 10^3$ CFU $g^{-1}$ (3.69 log CFU $g^{-1}$). As shown in the previous research, untreated maple pea spread with bruschetta reached the admissible level (3.69 log CFU $g^{-1}$) in less than four days of refrigerated storage (Kirse & Karklina, 2015; Kirse et al., 2016b).

In the present research, a similar trend was observed for the other three pulse spreads (cowpea spread, cowpea spread with seasoning and maple pea spread); the initial shelf-life of untreated pulse spreads was 3 to 5 days of refrigerated storage. Additionally, both *sous vide* and high pressure processed pouches ballooned after 1-week storage at room temperature (20 ± 1 °C) and TPC exceeded the admissible level (3.69 log CFU $g^{-1}$), therefore pulse spreads were only stored at 5 °C temperature. The short shelf-life of pulse spreads suggests that post-processing methods and appropriate packaging solutions should be considered to reduce the total number of microorganisms and avoid accelerated deterioration, thus extending shelf-life of the new products.

A preliminary study on the shelf-life extension of maple pea (*Pisum sativum* var. *arvense* L.) spread using *sous vide* treatment (80 °C/15 min) suggested the possible shelf-life of pea spread up to 96 days based on microbiological quality (Kirse et al., 2016b); the presence on anaerobic pathogens was not confirmed. However, microorganism growth during storage after *sous vide* treatment showed that microorganisms adapt to the environment in 50 to 60 days, and then a more rapid growth can be observed. Therefore, the investigated storage time for the processed pulse spreads in the current research was 62 days (proportional to the start of the accelerated growth).

A previous study showed that mean microorganism count in untreated maple pea spread with seasoning accounted to 3.41 log CFU $g^{-1}$ (Kirse et al., 2015). The current study established that TPC in untreated spreads ranged from 3.40 to 3.48 log CFU $g^{-1}$ without significant differences among samples ($p > 0.05$). An over 1.5-log reduction in

TPC compared to untreated spread samples was observed for pulse spreads after processing without significant differences between pulse spreads and samples in both packaging materials ($p < 0.05$). The presence of yeasts and moulds was not confirmed in any *sous vide* and high pressure processed samples.

TPC dynamics in *sous vide* treated pulse spreads during storage in different flexible packaging films showed that TPC after 62-day storage was significantly higher than immediately after processing ($p = 0.011$) (Fig. 1). Initial TPC in spreads packed in PA/PE after *sous vide* treatment was between 1.67 to 1.77 log CFU $g^{-1}$ whereas in spreads packed in PET/ALU/PA/PP – between 1.62 to 1.71 log CFU $g^{-1}$. After storage over one-log increase of TPC in spreads was observed with mean counts ranging from 2.81 to 2.95 log CFU $g^{-1}$, analysis of variance of the data indicated no significant differences among pulse spreads and samples in both packaging materials ($p < 0.05$); TPC did not exceed admissible level ($N_{max} < 3.69$ log CFU $g^{-1}$) for any of the samples.

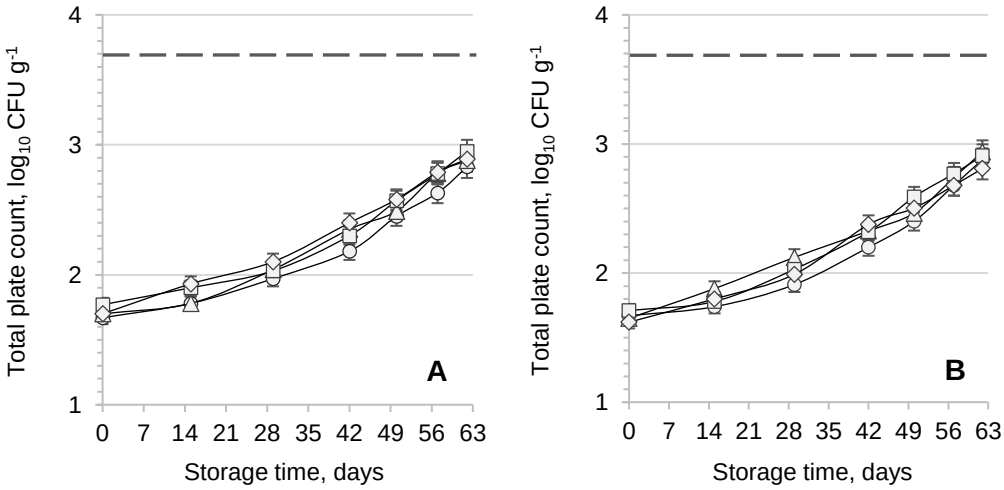



**Figure 1.** TPC dynamics during storage of *sous vide* treated cowpea spread (○), cowpea spread with bruschetta (△), maple pea spread (□) and maple pea spread with bruschetta (◇) packed in PA/PE (A) and PET/ALU/PA/PP (B). The dashed line (– –) denotes the admissible level of TPC < 3.69 log CFU $g^{-1}$ for vegetable purées (CM Regulation, 2014).

Available studies on the microbiological quality of products after *sous vide* treatment have been focussed mostly on meat processing. An investigation on *sous vide* processed beef (97 °C/11 min) carried out by Paik et al. (2006) found that samples inoculated with *B. cereus* and *C. perfringens* spore cocktail did not exceed the guidelines of bacterial loading after 60 days at 4 °C. Similar findings were given by Can & Harun (2015) who concluded that *sous vide* treated (90 °C/20 min) chicken meatballs maintain consistent microbiological quality for 70 days at 2 °C. Pino Hernández et al. (2017) pointed out that microbial load in *sous vide* treated (60 °C/9.48 min) fish fillets remained within the thresholds established by Brazilian laws during 63-day storage at 1 °C temperature. Whereas Levkane et al. (2010) showed that *sous vide* technology (65 °C/20 min) of potato salad with meat in mayonnaise was suitable to maintain microbiological

quality during 52-day storage at 4 ºC and 10 ºC temperature. The results of the present study are consistent with the findings of the above-mentioned investigations.

Total plate count dynamics in high pressure processed pulse spreads during storage in different flexible packaging films showed a similar trend to *sous vide* treated spreads – after 62-day storage TPC was significantly higher than in samples immediately after processing ($p$ = 0.017) (Fig. 2). Initial TPC in spreads packed in PA/PE after high pressure processing was between 1.78 to 1.87 log CFU $g^{-1}$ whereas in spreads packed in PET/ALU/PA/PP – between 1.75 to 1.87 log CFU $g^{-1}$. After storage over one-log increase of TPC in spreads was observed with mean counts ranging from 2.84 to 2.98 log CFU $g^{-1}$ without significant differences between pulse spreads and samples in both packaging materials ($p < 0.05$); TPC did not exceed admissible level ($N_{max} < 3.69$ log CFU $g^{-1}$) for any of the samples.

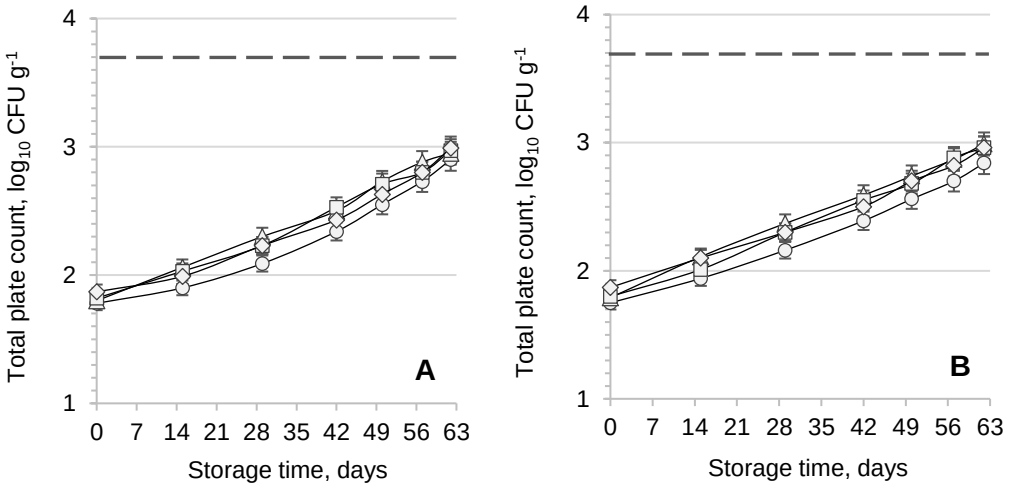



**Figure 2.** TPC dynamics during storage of high pressure processed cowpea spread (○), cowpea spread with bruschetta (△), maple pea spread (□) and maple pea spread with bruschetta (◇) packed in PA/PE (A) and PET/ALU/PA/PP (B). The dashed line (– –) denotes the admissible level of TPC < 3.69 log CFU $g^{-1}$ for vegetable purées (CM Regulation).

Similar to *sous vide* treatment, studies on the microbiological quality of products after high pressure processing as the post-processing treatment have been focussed on products of animal origin. Hygreeva & Pandey (2016) reviewed the studies of the last decade on high pressure processing of processed meat products, concluding that depending on the chosen processing regimes (400–600 MPa/3–10 min) and storage conditions (2–8 °C) high pressure processing ensures consistent microbiological quality throughout storage (40 to 120 days). Masegosa et al. (2015) found that pressure treatment (600 MPa/5 min) significantly reduced the mesophilic counts of ready-to-heat vegetable meals after processing and maintained their microbial stability throughout 20-day storage at 4 °C. Whereas, a study on the effect of different pressure levels (500 and 600 MPa/1 min) on lasagne ready meal indicated that high-pressure processing significantly reduced the total aerobic and lactic acid bacteria counts and prolonged the microbial shelf-life of lasagne for 56 days (Stratakos et al., 2015). The results of TPC

during storage of high pressure processed pulse spreads are consistent with the findings of the above described investigations.

The number of microorganisms during storage below the admissible level does not guarantee that the product is free from pathogens. Therefore, possible presence of pathogens was determined in all samples of pulse spreads stored at 5 ± 1 °C for both processing methods (Table 1). EC Regulation No 2073/2005 on microbiological criteria for vegetables, fruits and products thereof defines that the count of *E. coli* must be below 100 CFU $g^{-1}$ (n = 5, c = 2, m = 100 CFU $g^{-1}$, M = 1000 CFU $g^{-1}$), whereas CM Regulation No 461/2014 denotes that pathogens must be absent in 1 g of vegetable jams, purées and similar products. Results showed that the presence of such pathogens as *E. coli* and *C. perfringens* in pulse spreads before preservation treatment and after *sous vide* and high pressure processing with sequential storage for 62 days was not confirmed in any of studied packaging materials.

**Table 1.** Pathogens in *sous vide* and high pressure processed pulse spreads, CFU $g^{-1}$

| Microorganisms | Samples* | Before processing | After 62-day storage | |
|---|---|---|---|---|
| | | | *sous vide* treatment | high pressure processing |
| *Bacillus cereus* | CS | $2.00 \times 10^2$ | $3.60 \times 10^1$ | $4.10 \times 10^1$ |
| | CS_B | $2.41 \times 10^2$ | $6.80 \times 10^1$ | $6.70 \times 10^1$ |
| | MS | $2.02 \times 10^2$ | $5.80 \times 10^1$ | $5.70 \times 10^1$ |
| | MS_B | $1.96 \times 10^2$ | $2.30 \times 10^1$ | $3.20 \times 10^1$ |
| *Escherichia coli* | CS<br>CS_B<br>MS<br>MS_B | not detected | not detected | not detected |
| *Clostridium perfringens* | CS<br>CS_B<br>MS<br>MS_B | not detected | not detected | not detected |

* CS – cowpea spread, CS_B – cowpea spread with bruschetta,
MS – maple pea spread, MS_B – maple pea spread with bruschetta

Contamination level of *B. cereus* in pulse spreads after spread preparation was below $2.41 \times 10^2$ CFU $g^{-1}$, whereas after 62-day storage *B. cereus* accounted to $< 10^2$ CFU $g^{-1}$, indicating that chosen processing methods had a positive effect on *B. cereus* reduction in all samples. Insignificant differences were found between pulse spreads in different packaging materials ($p < 0.05$).

*B. cereus* is the dominant aerobic bacterium in cooked, pasteurized and chilled products, because of the probable survival of its spores during the pasteurization step after packing (Paik et al., 2006). Literature data suggests that only *B. cereus* levels between $10^5$ and $10^8$ cells and/or spores produce toxins that cause vomiting or diarrhoea (Ceuppens et al., 2015). The diarrhoeal toxin is produced in the temperature range of 10–43 °C, with an optimum of 32 °C whereas the production of emetic toxin is within the temperature range of 12–37 °C, with more toxin produced at 12–15 °C compared to higher temperatures (Finlay et al. 2000, Banerjee & Sarkar, 2004). During storage, the growth of *B. cereus* was not observed and *B. cereus* should not be a hazard when refrigeration is properly maintained throughout the shelf-life of pulse spreads, in order to limit the germination and multiplication of spores (Deák & Farkas, 2013). However,

even these low levels of *B. cereus* are not allowed according to the CM Regulation No 461/2014.

Microbiological parameters determine whether food products are safe for consumption, yet product flavour quality drives consumer acceptance and demand as argued by Singh-Ackbarali & Maharaj (2014), therefore sensory and physicochemical parameters need to be taken into consideration when predicting shelf-life of pulse spreads. Sensory properties of pulse spreads (overall appearance, aroma, mouthfeel and taste) during 62-day storage were evaluated by six experts and each spread sample was assigned a quality number (QN) on a 5-point scale (5 – very good quality to 1 – unsatisfactory quality) as described by Kirse et al. (2016d). The quality of *sous vide* treated pulse spreads was very good (QN = 4.96–4.75) up to day 29 regardless of packaging film material; cowpea spread and maple pea spread maintained good quality during 62-day storage in both chosen packaging films, whereas spreads with bruschetta maintained good quality up to day 57 and average quality – after day 57. High pressure processed cowpea spreads maintained very good quality during the whole storage time (QN = 4.99–4.79), besides packaging film materials and seasoning had insignificant influence on the quality of cowpea spreads. Maple pea spread (MS), however, maintained good quality after day 57; maple pea spread with bruschetta (MS_B) in PA/PE packaging maintained good quality, but in PET/ALU/PA/PP packaging – very good quality (Kirse et al., 2016d). With regards to physicochemical parameters, *sous vide* and high pressure processing had an insignificant influence on pH, water activity, mass losses (Kirse et al., 2016c) and nutritional value of pulse spreads (Kirse & Karklina, 2016) after processing and throughout storage, irrespective of packaging materials ($p > 0.1$). Considering that *sous vide* and high pressure processing maintained consistent physicochemical and microbiological quality of pulse spreads during 62-day storage, shelf-life prediction was based on the results of sensory evaluation.

As defined in EU legislation, Commission Regulation (EC) No. 2073/2005 defines shelf-life as 'either the period corresponding to the period preceding the 'use by' or the minimum durability date'. In Commission Regulation (EC) No. 1169/2011 minimum durability date is defined as the date 'until which the foodstuff retains its specific properties when properly stored' and should be preceded by the words 'best before ...' (when the date includes an indication of the day) or 'best before end ...'; whereas in cases where foodstuffs are highly perishable from the microbiological point of view and are 'therefore likely after a short period to constitute an immediate danger to human health, the date of minimum durability shall be replaced by the 'use by' date'. Any special storage conditions (e.g., temperature not to exceed 5 °C) must be specified on the packaging when using 'best before' date. Robertson (2010) reported that such practice allows manufacturers to set the quality standard of the food, as the product will still be acceptable to many consumers after the 'best before' date has passed. Based on the results of quality changes during storage the suggested shelf-life of processed pulse spreads is given in Table 2.

Processed pulse spreads should be given the 'minimum durability date' and packaging should contain 'best before ...' label, as both processing methods were able to suppress the survival of investigated microorganisms in pulse spreads and total plate count did not exceed the admissible level at day 62.

**Table 2.** Suggested shelf-life of pulse spreads

| Processing technology | Packaging | Pulse spreads* | | | |
|---|---|---|---|---|---|
| | | CS | CS_B | MS | MS_B |
| *Sous vide* treatment | PA/PE | 62 days | 57 days | 62 days | 57 days |
| | PET/ALU/PA/PP | 62 days | 57 days | 62 days | 57 days |
| High pressure processing | PA/PE | 62 days | 62 days | 62 days | 62 days |
| | PET/ALU/PA/PP | 62 days | 62 days | 62 days | 62 days |

* CS – cowpea spread, CS_B – cowpea spread with bruschetta,
MS – maple pea spread, MS_B – maple pea spread with bruschetta

## CONCLUSIONS

*Sous vide* (80 °C/15 min) and high pressure processing (700 MPa/10 min/20 °C) significantly reduced the total microorganism count in cowpea and maple pea spreads thus maintaining consistent microbiological quality for 62 days at 5 °C. The presence of such pathogens as *E. coli* and *C. perfringens* in pulse spreads was not confirmed, whereas the level of *B. cereus* spores found in all spread samples did not possess harm to consumers. Considering microbiological, sensory and physicochemical quality changes during storage, the suggested shelf-life of processed pulse spreads is 62 days, except for *sous vide* treated spreads with bruschetta in both packaging materials – 57 days. Both processing methods are suitable to ensure the production of high quality pulse spreads with significantly longer shelf-life compared to untreated spreads.

ACKNOWLEDGEMENTS. The research was supported by the 7th Research Framework Programme of the European Union, Research Project No 613781 'EUROLEGUME – Enhancing of legumes growing in Europe through sustainable cropping for protein supply for food and feed' (FP7-KBBE-2013-7).

## REFERENCES

Ates, M.B., Skipnes, D., Rode, T.M. & Lekang, O.I. 2016. Comparison of spore inactivation with novel agitating retort, static retort and combined high pressure-temperature treatments. *Food Control* **60**, 484–492.

Balasubramaniam, V.M. 2010. Food Preservation: High Pressure. In Heldman, D.R. & Moraru, C.I. (eds.): *Encyclopedia of Agricultural, Food, and Biological Engineering*. Taylor and Francis, New York, pp. 543–549.

Baldwin. D.E. 2012. Sous vide cooking: A review. *International Journal of Gastronomy and Food Science* **1**, 15–30.

Banerjee, M. & Sarkar, P.K. 2004. Growth and enterotoxin production by spore forming bacterial pathogens from spices. *Food Control* **15**(6), 491–496.

Brody, A.L., Bugusu, B., Han, J.H., Sand, C.K. & McHugh, T.H. 2008. Innovative food packaging solutions. *J. Food Sci.* **73**, 107–116.

Can, Ö.P. & Harun, F. 2015. Shelf-life of chicken meat balls submitted to *sous vide* treatment. *Rev. Bras. Cienc. Avic.* **17**(2), 137–144.

Ceuppens, S., Boon, N. & Uyttendaele, M. 2015. Diversity of *Bacillus cereus* group strains is reflected in their broad range of pathogenicity and diverse ecological lifestyles. *FEMS Microbiol. Ecol.* **84**(3), 433–450.

Church, I.J. & Parsons, A.L. 2000. The sensory quality of chicken and potato products prepared using cook-chill and *sous vide* methods. *Int. J. Food Sci. Tech.* **35**, 155–162.

Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. 2005. Official Journal of the European Union **L338**, 1–26.

Commission Regulation (EC) No 1169/2011 of 25 October 2011 on the provision of food information to consumers 2011. *Official Journal of the European Union* L304, 18.

Deák, T. & Farkas, J. 2013. *Microbiology of thermally preserved foods: canning and novel physical methods*. DEStech Publications, Lancaster, 318 pp.

Dermesonluoglu, E., Fileri, K., Orfanoudaki, A., Tsevdou, M., Tsironi, T. & Taoukis, P. 2016. Modelling the microbial spoilage and quality decay of pre-packed dandelion leaves as a function of temperature. *J. Food Eng.* **184**, 21–30.

Finlay, W.J.J., Logan, N.A. & Sutherland, A.D. 2000. *Bacillus cereus* produces most emetic toxin at lower temperatures. *Lett. Appl. Microbiol.* **31**, 385–389.

Georget, E., Sevenich, R., Reineke, K., Mathys, A., Heinz, V., Callanan, M., Rauh, C. & Knorr, D. 2015. Inactivation of microorganisms by high isostatic pressure processing in complex matrices: a review. *Innov. Food Sci. Emerg.* **27**, 1–14.

Hygreeva, D. & Pandey, M.C. 2016. Novel approaches in improving the quality and safety aspects of processed meat products through high pressure processing technology - A review. *Trends Food Sci. Tech*. 54, 175–185.

Juneja, V.K. & Snyder, O.P. 2007. *Sous vide* and cook-chill processing of foods: concept development and microbiological safety. In Tewari, G., Juneja, V.K. (eds.): *Advances in Thermal and Non-Thermal Food Preservation*. Blackwell Publishing, Iowa, pp. 145–163.

Keenan, D.F., Brunton, N., Butler, F., Wouters, R. & Gormley, R. 2011. Evaluation of thermal and high hydrostatic pressure processed apple purees enriched with prebiotic inclusions. *Innov. Food Sci. Emerg.* **12**(3), 261–268.

Kirse, A. & Karklina, D. 2015. Integrated evaluation of cowpea (*Vigna unguiculata* (L.) Walp.) and maple pea (*Pisum sativum* var. arvense L.) spreads. *Agronomy Research* **11**, 103–110.

Kirse, A. & Karklina, D. 2016. Influence of *sous vide* treatment and high pressure processing on nutritional value and overall acceptance of pulse spreads. In Jakobsone, I. & Jakobsone, M. (eds.): *Nutrition and Health. 2nd International Conference. Book of Abstracts*. University of Latvia Press, Riga, Latvia, pp. 121.

Kirse, A., Karklina, D. & Muizniece-Brasava, S. 2016a. Consumer acceptance of new pulse spreads before and after *sous vide* treatment. *J. Int. Sci. Publ.: Agric. Food* **4**, 104–114.

Kirse, A., Karklina, D. & Muizniece-Brasava, S. 2016b. Shelf life extension of maple pea (*Pisum sativum* var. *arvense* L.) spread using *sous vide*. *Proceedings of The Latvian Academy of Sciences. Section B* **70**(6), 393–399.

Kirse, A., Karklina, D., Muizniece-Brasava, S. & Galoburda, R. 2015. High pressure processing for pea spread shelf life extension: a preliminary study. *GRANT journal* **4**(2), 102–108.

Kirse, A., Karklina, D., Muizniece-Brasava, S. & Galoburda, R. 2016c. Physicochemical stability of pulse spreads during storage after *sous vide* treatment and high pressure processing. *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering* **10**(12), 719–723.

Kirse, A., Straumite, E. & Karklina, D. 2016d. Influence of *sous vide* treatment and high pressure processing on sensory quality of pulse spreads during storage. In: Gaile, Z. & Sterne, D. (eds): *Agricultural science for industry progress. Harvest Festival "Vecauce-2016". Scientific seminar proceedings.* Latvia University of Agriculture, Jelgava, Latvia, pp. 36–39 (in Latvian).

Levkane, V., Muizniece-Brasava, S. & Dukalska, L. 2010. *Sous Vide* Packaging Technology Application for Salad with Meat in Mayonnaise Shelf-life Extension. *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering* **4**(5), 345–349.

Masegosa, R., Delgado-Adamez, J., Contador, R., Sanchez-Iniguez, F. & Ramirez, R. 2015. Effect of processing by hydrostatic high pressure of two ready to heat vegetable meals and stability after refrigerated storage. *Food Sci. Technol. Int*. **20**, 605–615.

Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of presumptive *Bacillus cereus*: ISO 7932:2004 (2004) Available at: https://www.lvs.lv/lv/products/40951, 1 March 2017.

Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of presumptive *Escherichia coli*: ISO 7251:2005 (2005) Available at: https://www.lvs.lv/lv/products/54558, 1 March 2017.

Microbiology of food and animal feeding stuffs - Horizontal method for enumeration of *Clostridium perfringens*: ISO 7937:2004 (2004) Available at: https://www.lvs.lv/lv/products/16653, 1 March 2017.

Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products: ISO 6887-4:2003 (2003) Available at: https://www.lvs.lv/lv/products/31302, 1 March 2017.

Paik, H.D. , Kimb, H.J., Namb, K.J., Kim, C.J., Lee, S.E. & Lee, D.S. 2006. Effect of nisin on the storage of sous vide processed Korean seasoned beef. *Food Control* **17**(12), 994–1000.

Pino Hernández, E.J.G., Carvalho, R.N.D., Joele, M.R.S.P., Araújo, C.D.S. & Lourenço, L.D.F.H. 2017. Effects of modified atmosphere packing over the shelf life of *sous vide* from captive pirarucu (*Arapaima gigas*). *Innov. Food Sci. Emerg.* **39**, 94–100.

Rastogi, N.K., Raghavarao, K.S., Balasubramaniam, V.M., Niranjan, K. & Knorr, D. 2007. Opportunities and challenges in high pressure processing of foods. *Crit. Rev. Food Sci. Nutr.* **47**(1), 69–112.

Regulation No 461/2014 on requirements for food quality schemes, their implementation, operation, monitoring and control arrangements issued by the Cabinet of Ministers of the Republic of Latvia on 12 August 2014. Available from: http://likumi.lv/doc.php?id=268347, 1 March 2017 (in Latvian).

Robertson, G.L. 2010. Food Packaging and Shelf-life. In Robertson, G.L. (ed.): *Food Packaging and Shelf-life, A Practical Guide*. CRC Press, Boca Raton, pp. 1–16.

Sansone, L., Aldi, A., Musto, E.D., Amendola, E. & Mensitieri, G. 2012. Assesing the suitability of polylactic acid flexible films for high pressure pasteurization and sterilization of packaged foodstuff. *J. Food Eng.* **111**(1), 34–45.

Seydim, A.C., Acton, J. C., Hall, M.A. & Dawson, P.L. 2006. Effects of packaging atmospheres on shelf-life quality of ground ostrich meat. *Meat Sci.* **73**, 503–510.

Singh-Ackbarali, D. & Maharaj, R. 2014. Sensory Evaluation as a Tool in Determining Acceptability of Innovative Products Developed by Undergraduate Students in Food Science and Technology at The University of Trinidad and Tobago. *Journal of Curriculum and Teaching* **3**(1), 10–27.

Smelt, J.P. & Brul, S. 2014. Thermal inactivation of microorganisms. *Crit. Rev. Food Sci. Nutr.* **54**(10), 1371–1385.

Stratakos, A.C., Linton, M., Patterson, M.F. & Koidis, A. (2015) Effect of high-pressure processing on the shelf life, safety and organoleptic characteristics of lasagne ready meals during storage at refrigeration and abuse temperature. *Innov. Food Sci. Emerg*., **30**, 1–7.

Torres, J.A. & Velazquez, G. 2008. Hydrostatic pressure processing of foods. In Jun, S., Irudayaraj, J. (eds.): *Food Processing Operations Modeling: Design and Analysis*. CRC Press Inc., Florida, pp. 173–212.

Vercammen, A., Vanoirbeek, K.G.A, Lurquin, I., Steen, L., Goemaere, O., Szczepaniak, S., Paelinck, H., Hendrickx, M.E.G. & Michiels, C.W. 2011. Shelf-life extension of cooked

ham model product by high hydrostatic pressure and natural preservatives. *Innov. Food Sci. Emerg.* **12**, 407–415.

Yamani, M.I. & Mehyar, G.F. 2011. Effect of Chemical Preservatives on the Shelf-life of Hummus during Different Storage Temperatures. *Jordan Journal of Agricultural Sciences* **7**(1), 19–30.

Yordanov, D.G. & Angelova, G.V. 2010. High pressure processing for foods preserving. *Biotechnol. Biotech. Eq.* **24**(3), 1940–1945.

Zhang, H. & Mittal, G.S. 2008. Effects of High-Pressure Processing (HPP) on Bacterial Spores: An Overview. *Food Rev. Int.* **24**(3), 330–351.

*Agronomy Research* **15**(S2), 1316–1329, 2017

# Comparison of methods of extraction of phenolic compounds from American cranberry (*Vaccinium macrocarpon* L.) press residues

L. Klavins, J. Kviesis and M. Klavins*

University of Latvia, 19 Raina Blvd., LV–1586, Riga, Latvia
*Correspondence: maris.klavins@lu.lv

**Abstract.** American cranberries (*Vaccinium macrocarpon* L.) contain significant quantities of various phenolic compounds. Most of these compounds are recovered when berry juice is produced. However, a considerable part of polyphenols remain in berry press residues and are discarded as food industry waste. The aim of the study was to compare the methods of extraction of polyphenols (ultrasound, microwave-assisted, Soxhlet) from press residues of American cranberry. The impact of main extraction parameters (e.g., extraction time, solid/solvent ratio, solvent type) on the yield of extracted polyphenols. Ultrasound-assisted extraction showed the highest potential from all studied methods, given its fast, convenient use and low cost. Aqueous ethanol and methanol in the presence of acid (anthocyanin extractions should be assisted with trifluoroacetic acid, polyphenol extractions – with HCl) were assessed as the best solvents for extraction. The obtained extracts were characterised using the Folin-Ciocaulteu method for determination of total phenolics and the pH-differential method for determination of total anthocyanins, and UPLC–PDA was used to determine the content of individual anthocyanins. Cyanidin-3-*O*-arabinoside, peonidin-3-*O*-galactoside, peonidin-3-*O*-glucoside and peonidin-3-*O*-arabinoside were identified as the main anthocyanins in cranberry press residue extracts.

**Key words:** phenolic compounds, antioxidant activity, flavonoids, anthocyanins, *Vaccinium macrocarpon*, press residues

## INTRODUCTION

Fruits of American cranberry (*Vaccinium macrocarpon*) are a rich source of phenolic compounds, including flavonoids, phenolic acids and other biologically active substances (Vvedenskaya et al., 2004; White et al., 2010; Kylli, 2011a). The main flavonoids found in berries are anthocyanins, proanthocyanidins, flavonols and catechins (Ancilotti et al., 2016). Flavonoids are responsible for the red colour of fruits and are the most abundant phenolic compounds in various berries. The basic flavonoid structure encompasses the flavan nucleus, containing 15 carbon atoms arranged in three rings. Phenolic acids present in berries are hydroxylated derivatives of benzoic acid and cinnamic acid. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen and hydroxyl radical quenchers. Consumption of natural antioxidants and, inter alia, phenolic compounds is associated with a protective effect against many diseases, such as cardiovascular diseases, obesity, urinary tract diseases, cancer and other

degenerative disorders (Nile and Park, 2014). American cranberry, used fresh or in the form of preserves, juices, wines, liquors or extracts, is among the most consumed berries in the countries of Northern Europe, USA, Canada and Russia (Roopchand et al., 2013).

A widely used approach for the processing of American cranberry is the production of juice, resulting in food industry waste – berry press residues (pomace), containing berry skin and seeds. Due to its acidity and low protein content, it has limited use and usually is discarded (White et al., 2010). From the perspective of valorisation of food industry wastes, berry press residues are a promising source of natural antioxidants – phenolic compounds. Extraction of phenolic compounds from food by-products has been reported for apple pomace (Pingret et al., 2012), black chokeberry wastes (D'Alessandro et al., 2014), chicory grounds (Pradal et al., 2016) and bilberry press residues (Aaby et al., 2013; Kerbstadt et al., 2015). The composition of phenolic compounds in plant material depends on plant species and their distribution in different tissues. Large amounts of phenolic compounds are bound in berry seeds and skin, which makes the release of these compounds difficult. Therefore, an extraction method specifically for berry press residues should be developed. The extraction conditions provided for one plant cannot be directly used for the extraction of phenolics from another plant due to the specific localisation of phenolics in various species. To develop methods for industrial application, the optimisation of extraction conditions is needed.

The aim of the study was (1) to select the best method for the extraction of polyphenols – specifically, anthocyanin – from berry press residues of American cranberry, (2) to elucidate the impact of the main extraction parameters (extraction time, solid/solvent ratio, solvent type and others) on the yield of extracted polyphenols and (3) to identify the anthocyanin composition of American cranberry.

## MATERIALS AND METHODS

### Berry samples and their processing

Berries of American cranberry (*Vaccinium macrocarpon* L.) were harvested in autumn (September 2016). Cranberries were hand-picked at a commercial farm (Z/S 'Strēlnieki') located on the outskirts of Jūrmala City (Latvia). All berries were frozen at -20 °C to improve the release of juice. Berries were then gently thawed at 5 °C. Once thawed, they were put into a domestic hydraulic 6 L juice extractor (manufactured by Biowin®, Poland) and drained of all juice. At this step, berry press residues (seeds, skins) containing residual moisture (10%) were produced. Berry press cake was frozen once again at -20 °C to prepare it for lyophilisation. Frozen berries were freeze-dried for 3 days in a Labconco® FreeZone benchtop freeze dryer at -45 °C. Finally, dried berries were homogenised to a fine powder using an IKA® M20 analytical mill.

### Chemicals and reference substances

Ethanol, acetone (Enola*)*, methanol and acetonitrile (ChemPur) used for extractions were of analytical grade. Demineralised water was obtained from a Milli Q system (Millipore*)*. Trifluoroacetic acid (99.5%), formic acid (99.9%), acetic acid (99.9%), gallic acid (97%) and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich. Sodium carbonate, potassium chloride, sodium acetate, trichloroacetic acid (all of ≥99.5% purity) as well as hydrochloric acid (37%), and sulphuric acid (98%) were purchased from Enola. Methanol (99.9% HPLC grade, Sigma-Aldrich) used for UHPLC

and reference standards of cyanidin-3-*O*-galactoside (≥97%), cyanidin-3-*O*-glucoside (≥96%), cyanidin-3-*O*-arabinoside (≥95%) and peonidin-3-*O*-glucoside (≥95%) were purchased from Extrasynthese (France). Peonidin-3-*O*-galactoside (≥97%) and peonidin-3-*O*-arabinoside (≥97%) were obtained from Polyphenols Laboratories AS (Norway).

**Extraction methods**

Four extraction methods were tested for an impact on the anthocyanin/polyphenol yields. Microwave and ultrasound-assisted extractions were performed using 0.50 g of lyophilised, homogenised cranberry powder, and 1.0 g of berry powder was used for Soxhlet extraction. Microwave, ultrasound and maceration-assisted extractions were done using 50 mL of solvent, while Soxhlet extraction, due to the larger extraction volume needed, was done using 100 mL of solvent. After the extraction procedure, extracts were filtered through cellulose filter paper with a pore size of 20 μm to remove berry particles and insoluble matter and stored in dark at 4 °C.

*Soxhlet extraction*

Soxhlet extraction was performed using 100 mL of 96% ethanol and 0.5% trifluoroacetic acid (TFA) (v/v). Berry powder (1.0 g) was weighed into a cellulose thimble and set for the extraction at 80 °C for 12 hours with a condenser. 25 cycles were completed during the extraction period. A five-sample block heater was used for simultaneous extractions in a *Behr ET2* Soxhlet extraction unit (Labor-Technik).

*Microwave-assisted extraction*

Microwave-assisted extraction was done using a Milestone Ethos One microwave extraction unit. Samples were weighed (0.5 g) straight into the microwave extraction capsules, and 50 mL of appropriate solvent was added. The applied extraction programme consisted of 10-minute heat-up time at 600 W to reach 80 °C, in which, the sample solution was held for 20 minutes.

*Ultrasound-assisted extraction*

Extraction from cranberry press residues was optimised using the ultrasound-assisted extraction method. A berry sample (0.5 g) was weighed, and 50 mL of solvent was added. 100 W ultrasound was used for the optimisation experiments, and, to compare the impact of the capacity of ultrasound on the efficiency of extraction, an experiment with ultrasound of a higher capacity (360 W) was done (Cole-Parmer). The temperature of ultrasound bath was monitored and not allowed to exceed 30 °C, at which point the water was replaced (every 20 minutes). After the treatment with ultrasound, the samples were left shaking for 24h in the dark. For comparison, to show the efficiency of ultrasound treatment, an experiment was performed where a sample was left shaking for 24h without ultrasound treatment.

**Chemical analysis**

*Determination of dry residue*

To determine dry residue of each extract, a set of glass vials were dried at 80 °C overnight. The vials were cooled down in a desiccator for at least 3 hours prior to use. Each vial was then marked and weighed on analytical scales. 1 mL of each extract was measured with a volumetric pipette and transferred into a dry vial. The vials were dried at 40 °C overnight to evaporate the solvents and moisture. The vials with dry residue

were put into a desiccator and cooled down for at least 3 hours. After that, the vials were weighed. The difference in weight of the vial before and after the addition of extract was expressed as a dry residue of the extract of 100 $g^{-1}$ berry powder.

*Determination of total carbohydrates*

Total carbohydrates were determined by the phenol-sulphuric acid method using glucose as a standard (Dubois et al., 1956). 1 mL of the berry extract or 1 mL of an appropriate dilution of the berry extract was mixed with 1 mL of 5% (w/v) phenol solution. 5 mL of sulphuric acid was added to the mixture and carefully mixed by shaking. The samples were incubated for 40 minutes at room temperature and measured at 488 nm. A calibration curve was prepared in the same manner using glucose standard solutions (0–200 mg $L^{-1}$). All measurements were made using a *Shimadzu UV-1800 UV-VIS* spectrophotometer against a reagent blank, where the sample volume was substituted with de-mineralised water.

*Determination of total phenolic compounds (TPC)*

Total polyphenols were quantified using the Folin-Ciocalteu colorimetric method (Folin and Ciocalteu, 1927; Siriwoharn et al., 2004). A standard curve was prepared ($R^2$ = 0.999) using gallic acid in the range of 0–0.350 g $mL^{-1}$. Standards were prepared by solution in water and measured at 765 nm after a 20–30-minute incubation period at room temperature in the dark. Appropriate sample dilutions were prepared in the same manner as the standards and measured against a de-mineralised water blank. All measurements were made using a *Shimadzu UV-1800 UV-VIS* spectrophotometer. Total polyphenols in g of gallic acid equivalents (GAE) 100 $g^{-1}$ berry powder (BP) were calculated using the following equation (Eq. 1),

$$TPC\ g\ GAE\ 100\ g^{-1}\ BP = \frac{C.\ of gallic\ acid\ (g\ mL^{-1}) \times Volume\ of\ extract\ (mL)}{Weighed\ amound\ of\ the\ sample\ (g)} \times 100 \quad (1)$$

where: *C*. of gallic acid (g $mL^{-1}$) is calculated using a calibration curve regression equation.

*Determination of total anthocyanins*

The spectrophotometric pH-differential method (Lee et al., 2005) was used to determine the total amount of anthocyanins in the prepared extracts. In this method, the ability of anthocyanin molecules to change colour at different pH levels is used. Two buffer solutions with different pH were prepared: 0.025 M potassium chloride solution at pH 1.0 and 0.4 M sodium acetate solution at pH 4.5; pH was adjusted with concentrated hydrochloric acid (HCl). Two dilutions of the same sample were prepared using the buffers described above, so that the Abs at 520 nm falls within the linear range of the spectrophotometer (0.1–1.4 AU) and does not exceed the 1:5 sample/buffer ratio. The diluted samples were left in the dark for 20–30 minutes and measured within 20–40 minutes. The absorbance of each dilution was measured at 520 nm and 700 nm against a de-mineralised water blank using *Shimadzu UV-1800 UV-VIS* spectrophotometer. The total anthocyanin content was calculated using following equation (Eq. 2),

$$Anthocyanin\ content\ (cyanidin-3-glucoside\ eq.,\ g\ L^{-1} = \frac{A \times MW \times DF}{\varepsilon \times l} \quad (2)$$

where: A = ($A_{520nm} - A_{700nm}$) pH 1.0 – ($A_{520nm} - A_{700nm}$) pH 4.5; MW (molecular weight) = 449.2 g $mol^{-1}$ for cyanidin-3-glucoside; DF = dilution factor; l = cuvette path length in cm (1cm); $\varepsilon$ = 26 900 molar extinction coefficient, in L x $mol^{-1}$ x $cm^{-1}$ for cyd-3-glu.

The extraction yield in g of anthocyanin 100 $g^{-1}$ berry powder (BP) was calculated using following equation (Eq. 3),

$$g\ anthocyanin\ 100\ g^{-1}BP = \frac{Anthocyanin\ (g\ L^{-1}) \times Volume\ of\ extract(L)}{weighed\ amount\ of\ the\ sample\ (g)} \times 100 \quad (3)$$

*Ultra-high performance liquid chromatography analysis*

Ultra-performance liquid chromatography (UPLC) identification and quantification analyses of anthocyanins were carried out using a Waters ACQUITY UPLC system equipped with a Quaternary Solvent Manager (QSM), a Sample Manager – Flow-through Needle (cooled to 4 °C) (SM–FTN), a column heater (CH–A) and a photodiode array (PDA) λ detector. PDA data were collected using a Waters Empower data systems software.

The analyses were carried out at 35 °C using a C18 column (Acquity UPLC BEH C18 2.1×50 mm i.d., 1.7 μm) with a column pre-filter (frit and nut 0.2 μm and 2.1 mm). The mobile phase consisted of aqueous 5.0% formic acid (A) and methanol/1.0% formic acid in water (70:30 v/v) (B). The flow rate was 0.250 mL $min^{-1}$, and the gradient elution was from 80% to 75% of solvent A in 15 minutes, from 75% to 60% in 7 minutes and from 60% to 0% in 18 minutes, followed by 10 min of stabilisation at 80%. The total sample run time was 40 minutes. The injection volume for samples was 2.0 μL. Identity assignment was carried out considering the retention times and by PDA analysis. Anthocyanins were quantified using external calibration curves prepared form anthocyanin standard mixture (3–100 mg $L^{-1}$).

**Statistics and data analysis**

All measurements were made in triplicate and expressed as a mean. Measurement standard deviations were calculated for each result. Standard curves were prepared in MS Excel software in the linear range of measurements, with the correlation coefficient ($R^2$) of at least 0.999. Statistical (significance at $\alpha$ = 0.05) tests (*Student's t-test*, *ANOVA*, *Tukey's HSD*) and calculations were performed in JMP® (SAS) software for statistics.

## RESULTS AND DISCUSSION

Despite polyphenolic and anthocyanin extractions from different berries being intensively studied and performed, there is still substantial inconsistency in the way how it is done. Considering the differences in chemical and physical properties of different polyphenols, these polar molecules are usually extracted with methanol (Lätti et al., 2008; Corrales et al., 2010; Sójka et al., 2013; Wiczkowski et al., 2013), ethanol (Chen et al., 2007; d'Alessandro et al., 2012; Ćujić et al., 2016), acetone (Vatai et al., 2008; Kylli et al., 2010; Kylli et al., 2011b; Šliumpaitė et al., 2013; Chen et al., 2016), acetonitrile (Lätti et al., 2008; Li et al., 2011) or water (Kim et al., 2009; Denev et al., 2010; d'Alessandro et al., 2012). Despite methanol and acetone being the most effective extraction solvents, their use is limited in food industry due to toxicity. Ethanol, in turn,

is a solvent more suitable for food industry. To assist the extraction of anthocyanins, various acids are used, i.e. trifluoroacetic acid (Li et al., 2011; Wiczkowski et al., 2013), HCl (Burdulis et al., 2007; Chen et al., 2007), formic acid (Lätti et al., 2008; Sójka et al., 2013), citric acid (Denev et al., 2010) and acetic acid (Chen et al., 2016). The addition of acids in anthocyanin extraction stabilises these molecules in the flavylium cation form, which produces red colour at low pH. The choice of acid can influence the stability of anthocyanins. For example, hydrochloric acid (HCl) can catalyse hydrolysis of acetylated anthocyanins. Therefore, organic acids are preferred for this type of extraction (Denev et al., 2010).

**Selection of solvent for polyphenol extraction**

Various extraction conditions suggested in other studies were considered in order to compare the different solvent systems used for the extraction of phenolic compounds. The suggested solvent mixtures were tested on the same type of sample, i.e. press residues of American cranberry, to find the best solvent for the specific type of sample used in this study. Ultrasound-assisted extraction for 40 minutes was used for each sample. The content of dry residue, which indicates the overall efficiency of extraction, showed variations among the different solvents used. For example, water and 1% HCl extraction shows significantly lower extraction yields ($\alpha$ = 0.05) in all the measured parameters, except total carbohydrates (14.82 g 100 $g^{-1}$ berries). Thus, considering the application of the extract, a solvent system where water is used should be avoided, as the low levels of phenolics (0.89 g 100 $g^{-1}$ berries) and anthocyanins (0.098 g 100 $g^{-1}$ berries) and high levels of total carbohydrates might not be applicable for further analytical study of extract composition. As the extracts obtained in this study were analytically characterised using liquid chromatography, the amount of total carbohydrates (7.82–19.52 g 100 $g^{-1}$ berries) does not interfere with the methods used to characterise them. However, if extracts are intended to be used for production purposes, the high amounts of sugars might be an inconvenience, as sugars make the final product a thick, viscous mass, which could be hard to handle and process (Table 1).

**Table 1.** Comparison of different solvent mixtures used for the extraction of phenolic compounds/anthocyanins. Uncertainty represents standard deviation. All solvents were used as v $v\%^{-1}$. Asterisk (*) represents a significant difference in the results ($p \leq 0.05$, Student's t-test)

| Solvent | Dry residue, g 100 $g^{-1}$ berry powder | Total carbohydrates, g 100 $g^{-1}$ berry powder | Anthocyanins, g 100 $g^{-1}$ berry powder | Total polyphenols, g 100 $g^{-1}$ berry powder |
|---|---|---|---|---|
| Acetonitrile 49.5%, TFA 0.5%, water 50% | 37.24 ± 1.53 | 7.82 ± 0.27* | 0.228 ± 0.006 | 3.84 ± 0.12* |
| Acetone 50% | 34.29 ± 1.41 | 12.17 ± 0.43 | 0.151 ± 0.004 | 2.70 ± 0.08 |
| Acetone 75% | 36.01 ± 1.48 | 18.52 ± 0.65* | 0.156 ± 0.004 | 2.69 ± 0.08 |
| Methanol 60%, acetone 30%, water 10% | 37.94 ± 1.56 | 16.86 ± 0.59 | 0.184 ± 0.005 | 2.34 ± 0.07 |
| Methanol, HCl 1% | 48.38 ± 1.98* | 17.93 ± 0.63 | 0.451 ± 0.011* | 4.80 ± 0.14* |
| Water, HCl 1% | 16.91 ± 0.69* | 14.82 ± 0.52 | 0.098 ± 0.002* | 0.89 ± 0.03* |
| Ethanol 70%, HCl 1% | 39.62 ± 1.59 | 16.85 ± 0.51 | 0.204 ± 0.005 | 3.43 ± 0.09 |

In our experiments, the highest extraction yields (48.38 g 100 $g^{-1}$ berries) were obtained by the use of methanol and 1% HCl (v/v). This extraction also gave the highest amount of total anthocyanins (0.451 g 100 $g^{-1}$ berries) and polyphenols (4.8 g 100 $g^{-1}$ berries). However, the stability of anthocyanin molecules must be considered when using this system. The easy use and low costs of ethanol and the high phenolic yield obtained from the use of this solvent (3.43 g 100 $g^{-1}$ berries) support the selection of lower alcohols (ethanol, methanol) for further optimisation of extraction (Table 1). Increasing varieties of products containing berry press residues are available for consumers. In this situation, there is a need for efficient extraction strategies to control the quality of such products.

**Comparison of polyphenol extraction methods**

The most often used polyphenol extraction methods are ultrasound-assisted extraction (Chen et al., 2007; Lätti et al., 2008; Ghafoor et al., 2009; Ćujić et al., 2016) and extraction where the sample is shaken in the extraction solvent for extended periods of time (Pinelo et al., 2005; Makris et al., 2008; Yi et al., 2009; Denev et al., 2010).

In addition to the two most often used methods, microwave-assisted extraction and Soxhlet extraction were also tested, as these methods prove to be reliable for routine use and produce repeatable results. All extractions were done with 96% ethanol and 0.5% TFA, v/v. Soxhlet and microwave extractions gave significantly lower overall yields than ultrasound and shaking-assisted extractions (23.88 g and 21.01 g 100 $g^{-1}$ berries, respectively). In addition, the latter two methods also gave lower levels of extracted anthocyanins (0.054 and 0.065 g 100 $g^{-1}$ berries) and polyphenols (1.21 g and 1.09 g 100 $g^{-1}$ berries) (Table 2). Soxhlet extraction is primarily performed with a solvent (ethanol) rather than acid, as trifluoroacetic acid forms an azeotropic mixture with water in ethanol, increasing the boiling point of the mixture and not allowing the acid to come in contact with the sample. Ultrasound-assisted extractions at different capacities (100 W or 360 W) showed similar extraction yields (34.05–34.53 g 100 $g^{-1}$ berries), total carbohydrates (11.46–12.15 g 100 $g^{-1}$ berries), anthocyanins (0.136–0.147 g 100 $g^{-1}$ berries) and polyphenols (1.59–1.68 g 100 $g^{-1}$ berries) (Table 2).

**Table 2**. Comparison of different extraction methods. All extractions were done with 96% ethanol and 0.5% TFA, v/v. Uncertainty represents standard deviation. Asterisk (*) represents significant difference in the results ($p \leq 0.05$, *Student's t-test*).

| Method | Dry residue, g 100 $g^{-1}$ berry powder | Total carbohydrates g 100 $g^{-1}$ berry powder | Anthocyanins, g 100 $g^{-1}$ berry powder | Total polyphenols, g 100 $g^{-1}$ berry powder |
|---|---|---|---|---|
| Microwave | 21.01 ± 0.86* | 8.80 ± 0.36* | 0.054 ± 0.001* | 1.09 ± 0.04* |
| Soxhlet | 23.88 ± 1.80* | 8.33 ± 0.34* | 0.065 ± 0.002* | 1.21 ± 0.05 |
| 100W ultrasound | 34.05 ± 1.40 | 11.46 ± 0.47 | 0.135 ± 0.003 | 1.59 ± 0.07 |
| 360W ultrasound | 34.53 ± 1.42 | 12.15 ± 0.50 | 0.147 ± 0.004 | 1.68 ± 0.07 |
| 24h shaking | 33.01 ± 1.35 | 11.78 ± 0.48 | 0.098 ± 0.002 | 1.12 ± 0.06* |

When the sample is treated with ultrasound, the cell wall matrix is disrupted, which ensures the release of various compounds, including polyphenols, into the surrounding medium, which is very important in this case, as the type of the sample (berry skins,

seeds) has thick cell walls (Ćujić et al., 2016). The results obtained from ultrasound-assisted extractions showed that this method has the greatest potential from all of the methods used. Given its fast, convenient use and low cost, it is the method of choice for phenolic extractions from berry press residues.

**Selection of acid for polyphenol extractions**

The extraction of polyphenolic substances is influenced not only by the choice of solvent (Table 1) but also the choice of acid used to assist the extraction. To identify the most optimal acid for the extraction of anthocyanin and phenolics, a series of ultrasound-assisted extractions were performed, where the solvent (96% ethanol) was mixed with various acids (at 1%, v/v) used for polyphenol extractions and routine work in a laboratory (Chen et al., 2007; Denev et al., 2010; Li et al., 2011; Sójka et al., 2013; Chen et al., 2016).

Extractions with no added acid, formic acid, acetic acid and sulphuric acid gave similar results for anthocyanins (0.122–0.145 g 100 $g^{-1}$ berries) and polyphenols (1.1–1.4 g 100 $g^{-1}$ berries) (Fig. 1).

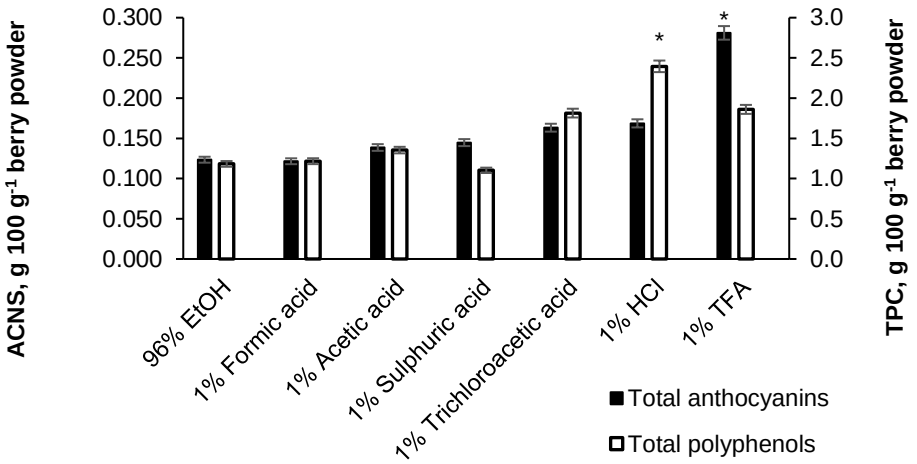



**Figure 1.** Comparison of total polyphenol (TPC) and total anthocyanin (ACNS) extraction efficiency using various acids at the concentration of 1% with 96% ethanol (v/v). Error bars represent 95% confidence interval. Asterisk (*) represents a significant difference in the results (*ANOVA, Tukey's HSD*).

Formic acid is one of the acids most often used in the extraction of anthocyanins. However, the extraction yields are lower in this solvent/acid system (no significant difference from 96% ethanol and no acid extraction) compared to using other acids. Another acid frequently used in the extraction of anthocyanins is trifluoroacetic acid (TFA), although at lower concentrations (0.1% and 0.5%). When using 1% TFA, the detected anthocyanin yield was approximately two times higher (0.280 g 100 $g^{-1}$ berries) than that of the extractions with carboxylic acids and sulphuric acid. The phenolic compounds extracted with 1% TFA were in similar concentrations to those extracted with trichloroacetic acid (1.9 g and 1.8 g 100 $g^{-1}$ berries, respectively). However, due to the difficult removal of trichloroacetic acid from the extracts, the use of this acid might

be problematic. The highest amount of extracted polyphenols was observed when using HCl (2.4 g 100 $g^{-1}$ berries). At the same time, the amount of extracted anthocyanins (0.169 g 100 $g^{-1}$ berries) was comparable with sulphuric acid and trichloroacetic acid extractions, indicating that HCl assists the extraction of non-anthocyanin phenolics (Fig. 1). Overall, the use of acids to increase the yields of polyphenol extractions is a necessary step. Taking into consideration that the acids used will not interfere with the analytical measurements, anthocyanin extractions should be assisted with trifluoroacetic acid and polyphenol extractions – with HCl.

**Ultrasound treatment kinetics**

Comparing the prospective extraction methods, it was concluded that ultrasound extractions give the highest yields of phenolics (Table 2) at the two different ultrasound capacities used. To optimise the procedure of ultrasound-assisted extraction, the duration of ultrasound treatment was investigated as one of the main factors influencing the extraction efficiency. A series of 7 experiments were done, taking three different concentrations of ethanol (96%, 40% and 70%) and 5% formic acid (v/v) and performing ultrasound-assisted (100 W) extraction for 3, 5, 8, 10, 15, 25 and 40 minutes. Two parameters – total anthocyanins and total polyphenols – were measured for each time-point. The amount of total anthocyanins was plotted against the ultrasound treatment time, revealing that 40% and 70% ethanol extractions give higher extraction yield than the extractions done with 96% ethanol (Fig. 2).

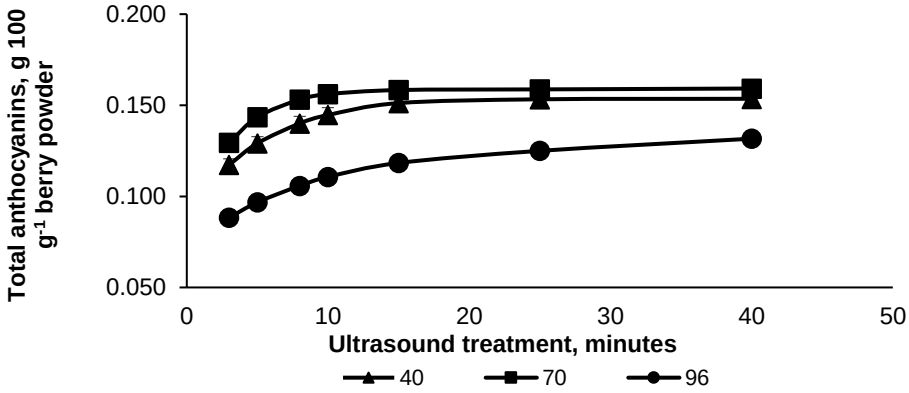



**Figure 2.** Total extracted anthocyanins from cranberry press residues depending on the duration of ultrasound treatment. Three different ethanol concentrations were used with 5% formic acid (v/v). Error bars represent 95% confidence interval.

The same pattern was also observed with the total polyphenols (Fig. 3). The amount of total anthocyanins extracted with 96% ethanol was significantly lower than that of the 40% and 70% ethanol extractions. The extraction kinetics revealed that the amount of extracted anthocyanins and polyphenols did not increase after 15 min of ultrasound treatment. In fact, there was no significant difference between the 15-, 25- and 40-minute time points as well as the 40% and 70% ethanol extractions (Figs 2 and 3). These results suggest that, first, the extraction solvent should contain water to increase the efficiency

of extraction of both anthocyanins and polyphenols, and, second, the optimal length of ultrasound treatment is 15–25 minutes.

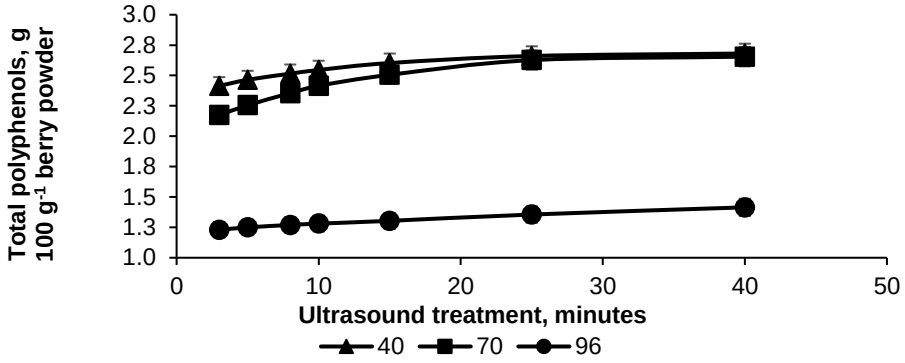



**Figure 3.** Total extracted polyphenols from cranberry press residues depending on the duration of ultrasound treatment. Three different ethanol concentrations were used with 5% formic acid (v/v). Error bars represent 95% confidence interval.

**Optimisation of solid/solvent ratio**

Maximisation of the total yield of extractions is an important step in optimisation. Not only it saves time but also resources, as the same result can be achieved by using less solvents and sample material. To investigate the capacity of ethanol and methanol for polyphenol extraction, a set of experiments with different berry press residue/solvent ratios was done. Four different solid/solvent ratios were tested: 1:30, 1:60, 1:90 and 1:120. In the experiments where ethanol was used, no significant difference could be seen between the different solid/solvent ratios. However, in the extractions where methanol was used, the optimal solid/solvent ratio was between 1:90 and 1:120, as these experiments resulted in significantly higher amounts of extracted anthocyanins (0.174 g and 0.169 g 100 $g^{-1}$ berries) and total polyphenols (2.34 g and 2.29 g 100 $g^{-1}$ berries) (Fig. 4).

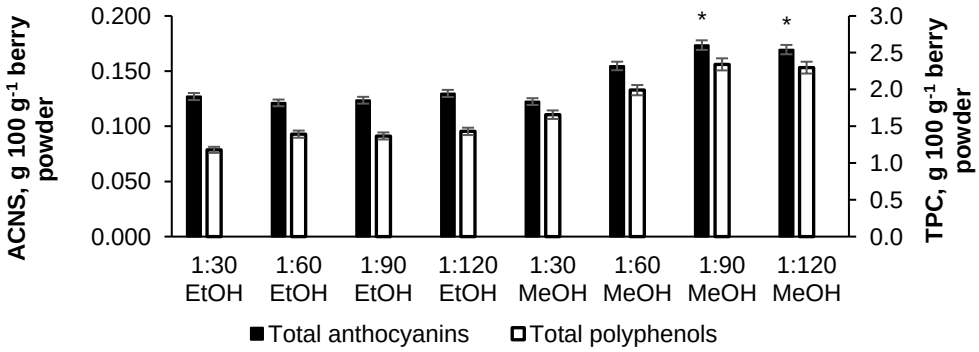



**Figure 4.** Effect of the solvent/solid ratio on the extraction of total polyphenol (TPC) and total anthocyanin (ACNS) using ethanol (EtOH) and methanol (MeOH) with 5% formic acid (v/v). Error bars represent 95% confidence interval. Asterisk (*) represents a significant difference in the results ($p \leq 0.05$, *Student's t-test*).

**Chromatographic identification of anthocyanins in cranberry extracts**

To characterise the composition of anthocyanins in the obtained extracts, UPLC-PDA chromatography was used. Considering the potential application and significance of anthocyanin extracts that have influence on human health, the anthocyanins found in cranberry extracts were identified and quantified.

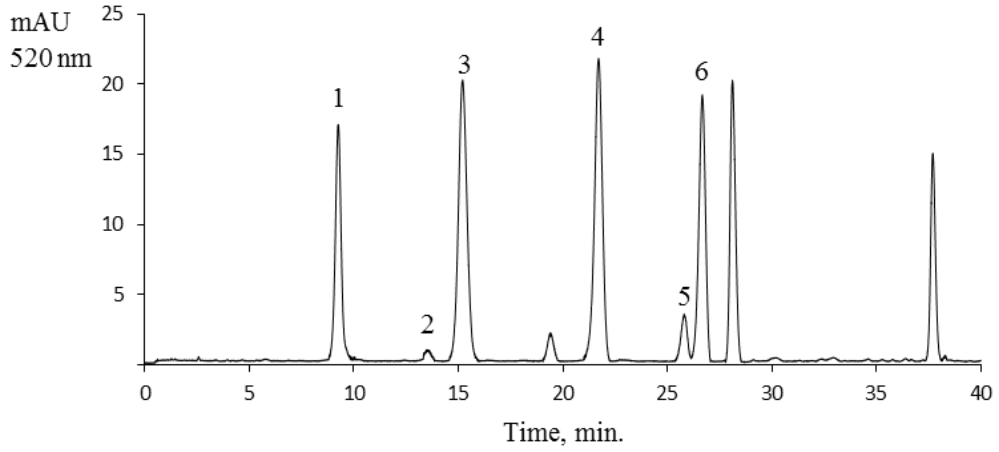



**Figure 5.** UPLC chromatogram (520 nm) of 96% ethanol, 5% formic acid (v/v) cranberry press residue extract. Peak numbers represent the anthocyanins quantified in Table 3.

Chromatographic analysis revealed the presence of a total of 9 major substances, and 6 of them were identified and quantified by comparison with reference substances (Fig. 5, Table 3). Cyanidin-3-*O*-arabinoside, peonidin-3-*O*-galactoside, peonidin-3-*O*-glucoside and peonidin-3-*O*-arabinoside were found in the highest concentrations. Similar grouping of anthocyanins in cranberries has been found in the previous studies with whole berries (Vvedenskaya et al., 2004), but the total concentrations of anthocyanins obtained in our study were higher than those found by other researchers, thus supporting the applicability of the extraction conditions used in this study.

**Table 3.** Profiling of anthocyanins in cranberry press residue extract by UPLC/PDA (selective wavelength 520 nm). Retention time (Rt) on PDA chromatogram.

| Peak No. | Compound name | Rt, min. | $\lambda_{max}$, nm | Amount, mg $g^{-1}$ dry powder) |
|---|---|---|---|---|
| 1 | cyanidin-3-*O*-galactoside | 9.26 | 278.15; 512.50 | 1.73 ± 0.17 |
| 2 | cyanidin-3-*O*-glucoside | 13.61 | 278.15; 328.11; 516.15 | 0.06 ± 0.01 |
| 3 | cyanidin-3-*O*-arabinoside | 15.21 | 278.15; 513.72 | 3.07 ± 0.31 |
| 4 | peonidin-3-*O*-galactoside | 21.71 | 278.15; 512.50 | 3.04 ± 0.21 |
| 5 | peonidin-3-*O*-glucoside | 25.81 | 278.15; 361.22; 514.93 | 0.36 ± 0.04 |
| 6 | peonidin-3-*O*-arabinoside | 26.68 | 278.15; 517.34 | 2.31 ± 0.23 |

[a] Data expressed as mean values ± standard deviation ($n$ = 3), mg $g^{-1}$ berry powder.

## CONCLUSIONS

The present study determined the most optimal extraction parameters for the extraction of polyphenols and anthocyanins from press residues of American cranberry. Several solvent/acid combinations, solid/solvent ratios and extraction methods were examined.

The performed experiments revealed that the use of ethanol and methanol with 1% trifluoroacetic acid for the extraction of anthocyanins and 1% HCl for the extraction of non-anthocyanin polyphenols are the optimal solvents for extraction. Ultrasound-assisted extraction with the ultrasound treatment for 15–25 minutes gave the highest extraction yields when the solid/liquid ratio was between 1:90 and 1:120. The results obtained in this study provide a reliable and repeatable method for the extraction of polyphenols and anthocyanins from press residues of American cranberries and, possibly, other berry powders.

ACKNOWLEDGEMENTS. This work was supported by the European Regional Development Fund within the project No. 1.1.1.1/16/A/047 'Genus Vaccinium berry processing using 'green' technologies and innovative, pharmacologically characterized biopharmaceutical products'.

## REFERENCES

Aaby, K., Grimmer, S. & Holtung, L. 2013. Extraction of phenolic compounds from bilberry (*Vaccinium myrtillus L.*) press residue: effects on phenolic composition and cell proliferation. *LWT-Food Science and Technology* **54**, 257–264.

d'Alessandro L.G., Kriaa K., Nikov I. & Dimitrov K. 2012. Ultrasound assisted extraction of polyphenols from black chokeberry. *Separation and Purification Technology* **93**, 42–47.

d'Alessandro, L.G., Dimitrov, K., Vauchel, P. & Nikov, I. 2014. Kinetics of ultrasound assisted extraction of anthocyanins from *Aronia melanocarpa* (black chokeberry) wastes. *Chemical Engineering Research and Design*, **92**, 1818–1826.

Ancilotti, C., Ciofi L., Pucci, D., Sagona, E., Giordani, E., Biricolti, S., Gori, M., Petrucci, W.A., Giardi, F., Bartoletti, R., Chiuminatto, U., Orlandini, S., Mosti, S. & Del Bubba, M. 2016. Polyphenolic profiles and antioxidant and antiradical activity of Italian berries from *Vaccinium myrtillus* L and *Vaccinium uliginosum* L subsp. *Gaultherioides* (Bigelow) S.B.Young. *Food Chemistry* **204**, 176–184.

Burdulis, D., Ivanauskas, L., Dirse, V., Kazlauskas, S. & Razukas, A. 2007. Study of diversity of anthocyanin composition in bilberry (*Vaccinium myrtillus* L.) fruits. *Medicina (Kaunas)* **43**(12), 971–977.

Chen, M.H., McClung, A.M. & Bergman, C.J. 2016. Concentrations of oligomers and polymers of proanthocyanidins in red and purple rice bran and their relationships to total phenolics, flavonoids, antioxidant capacity and whole grain color. *Food Chemistry* **208**, 279–287.

Chen, F., Sun, Y., Zhao, G., Liao, X., Hu, X., Wu, J. & Wang, Z. 2007. Optimization of ultrasound-assisted extraction of anthocyanins in red raspberries and identification of anthocyanins in extract using high-performance liquid chromatography–mass spectrometry. *Ultrasonics Sonochemistry* **14**(6), 767–778.

Corrales, M., Fernandez, A., Pinto, M.G.V., Butz, P., Franz, C.M., Schuele, E. & Tauscher, B. 2010. Characterization of phenolic content, in vitro biological activity, and pesticide loads of extracts from white grape skins from organic and conventional cultivars. *Food and Chemical Toxicology* **48**(12), 3471–3476.

Ćujić, N., Šavikin, K., Janković, T., Pljevljakušić, D., Zdunić, G. & Ibrić, S. 2016. Optimization of polyphenols extraction from dried chokeberry using maceration as traditional technique. *Food Chemistry* **194**, 135–142.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A.T. & Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**(3), 350–356.

Denev, P., Ciz, M., Ambrozova, G., Lojek, A., Yanakieva, I. & Kratchanova, M. 2010. Solid-phase extraction of berries' anthocyanins and evaluation of their antioxidative properties. *Food Chemistry* **123**(4), 1055–1061.

Folin, O., & Ciocalteu, V. 1927. On tyrosine and tryptophane determinations in proteins. *Journal of Biological Chemistry* **73**(2), 627–650.

Ghafoor, K., Choi, Y.H., Jeon, J.Y. & Jo, I.H. 2009. Optimization of ultrasound-assisted extraction of phenolic compounds, antioxidants, and anthocyanins from grape *(Vitis vinifera*) seeds. *Journal of Agricultural and Food Chemistry* **57**(11), 4988–4994.

Kim, Y.H., Bang, C.Y., Won, E.K., Kim, J.P. & Choung, S.Y. 2009. Antioxidant activities of *Vaccinium uliginosum* L. extract and its active components. *Journal of Medicinal Food* **12**(4), 885–892.

Kerbstadt, S., Eliasson, L., Mustafa, A. & Ahrne L. 2015. Effect of novel drying techniques on the extraction of anthocyanins from bilberry press cake using supercritical carbon dioxide. *Innovative Food Science and Emerging Technologies* **29**, 209–214.

Kylli, P. 2011a. Berry phenolics: isolation, analysis, identification and antioxidant properties. PhD thesis, Helsinki University: Helsinki.

Kylli, P., Nohynek, L., Puupponen-Pimiä, R., Westerlund-Wikström, B., Leppänen, T., Welling, J. & Heinonen, M. 2011b. Lingonberry (*Vaccinium vitis-idaea*) and European cranberry (*Vaccinium microcarpon*) proanthocyanidins: isolation, identification, and bioactivities. *Journal of Agricultural and Food Chemistry* **59**(7), 3373–3384.

Kylli, P., Nohynek, L., Puupponen-Pimiä, R., Westerlund-Wikström, B., McDougall, G., Stewart, D. & Heinonen, M. 2010. Rowanberry phenolics: compositional analysis and bioactivities. *Journal of Agricultural and Food Chemistry* **58**(22), 11985–11992.

Lätti, A.K., Riihinen, K.R. & Kainulainen, P.S. 2007. Analysis of anthocyanin variation in wild populations of bilberry (*Vaccinium myrtillus* L.) in Finland. *Journal of Agricultural and Food Chemistry* **56**(1), 190–196.

Lee, J., Durst, R.W. & Wrolstad, R.E. 2005. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. *Journal of AOAC International* **88**(5), 1269–1278.

Li, R., Wang, P., Guo, Q.Q. & Wang, Z.Y. 2011. Anthocyanin composition and content of the *Vaccinium uliginosum* berry. *Food Chemistry*, **125**(1), 116–120.

Makris, D.P., Boskou, G., Chiou, A. & Andrikopoulos, N.K. 2008. An investigation on factors affecting recovery of antioxidant phenolics and anthocyanins from red grape (*Vitis vinifera* L.) pomace employing water/ethanol-based solutions. *American Journal of Food Technol*. **3**(3), 164–173.

Nile, S.H. & Park, S.W. 2014. Edible berries: bioactive compounds and their effect on human health. *Nutrition* **30**, 134–144

Pingret, D., Fabiano-Tixier, A.S., Bourvellec, C.L., Renard, C.M.G.C. & Chemat, F. 2012. Lab and pilot scale ultrasound assisted water extraction of polyphenols from apple pomace. *Journal of Food Engineering* **111**, 73–81.

Pinelo, M., Rubilar, M., Jerez, M., Sineiro, J. & Núñez, M.J. 2005. Effect of solvent, temperature, and solvent-to-solid ratio on the total phenolic content and antiradical activity of extracts from different components of grape pomace. *Journal of Agricultural and Food Chemistry* **53**(6), 2111–2117.

Pradal, D., Vauchel, P., Decossin, S., Dhulster, P. & Dimitrov, K. 2016. Kinetics of ultrasound assisted extraction of antioxidant polyphenols from food by-products: extraction and energy consumption. *Ultrasonics Sonochemistry* **32**, 137–146.

Roopchand, D.E., Krueger, C.G., Moskal, K., Fridlender, B., Lila, M.A. & Raskin, I. 2013. Food-compatible method for the efficient extraction and stabilization of cranberry pomace polyphenols. *Food Chemistry* **141**(4), 3664–3669.

Siriwoharn, T., Wrolstad, R.E., Finn, C.E. & Pereira, C.B. 2004. Influence of cultivar, maturity, and sampling on blackberry (*Rubus* L. Hybrids) anthocyanins, polyphenolics, and antioxidant properties. *Journal of Agricultural and Food Chemistry* **52**(26), 8021–8030.

Šliumpaitė, I., Murkovic, M., Zeb, A. & Venskutonis, P.R. 2013. Antioxidant properties and phenolic composition of swallow-wort (*Vincetoxicum lute*a L.) leaves. *Industrial Crops and Products* **45**, 74–82.

Sójka, M., Kołodziejczyk, K. & Milala, J. 2013. Polyphenolic and basic chemical composition of black chokeberry industrial by-products. *Industrial Crops and Products* **51**, 77–86.

White, B.L., Howard, L.R. & Prior R.L. 2010 Proximate and polyphenolic characterization of cranberry pomace. *Journal of Agricultural and Food Chemistry* **58**, 4030–4036.

Vvedenskaya, I.O., Rosen, T.R., Guido, J.E., Russell, D.J., Mills, K.A. & Vorsa, N. 2004. Characterization of flavonols in cranberry (*Vaccinium macrocarpon*) powder. *Journal of Agricultural and Food Chemistry* **52**, 188–195.

Vatai, T., Škerget, M., Knez, Ž. Kareth, S., Wehowski, M. & Weidner, E. 2008. Extraction and formulation of anthocyanin-concentrates from grape residues. *The Journal of Supercritical Fluids* **45**(1), 32–36.

Wiczkowski, W., Szawara-Nowak, D. & Topolska, J. 2013. Red cabbage anthocyanins: Profile, isolation, identification, and antioxidant activity. *Food Research International* **51**(1), 303–309.

Yi, C., Shi, J., Kramer, J., Xue, S., Jiang, Y., Zhang, M. & Pohorly, J. 2009. Fatty acid composition and phenolic antioxidants of winemaking pomace powder. *Food Chemistry* **114**(2), 570–576.

*Agronomy Research* **15**(S2), 1330–1347, 2017

# Meat industry by-products for berry crops and food production quality improvement

M. Kremenevskaya[1], O. Sosnina[1], A. Semenova[2], I. Udina[3] and A.Glazova[1,*]

[1]ITMO University, Faculty of Food Biotechnologies and Engineering, Department of Meat and Fish Processing and Refrigeration, 49 Kronverksky Pr., St. Petersburg, 197101, Russia
[2]The Gorbatov's All-Russian Meat Research Institute (VNIIMP), Deputy director for scientific work, 26 Talalikhina Str., Moscow, 109316, Russia
[3]LLC JTI Russia, Scientific and Regulatory Affairs Department, Scientific and Regulatory Affairs Manager, 1st Krasnogvardeysky proezd, Moscow, 123100, Russia
*Correspondence: sosnina.olga.ITMO@yandex.ru

**Abstract.** This paper describes the problem of obtaining a hydrolysate from animal industry by-products. A new innovative protein-containing product has been created to stimulate the growth and development of berry and fruit crops. The paper describes a technique for a plant treatment with a hydrolysate invented, its concentrations being determined. We have studied the chemical composition of fruit and berry raw materials in a native form after rapid freezing and refregiration. The possibility of creating a new confectionery product made from quick-frozen berries treated with a stimulator is predetermined**.**

**Key words:** protein hydrolysate, stimulator of plant growth and development, quick-frozen berries, anthocyanins

## INTRODUCTION

Beef cattle farming can be made more cost-effective by complex meat by-products and waste processing. This will decrease the meat waste volume, which improve environmental situation and broaden the range of food and feed products available on the market. New products with tailored properties for a particular application can be produced in presence of chemical catalyst in micro concentration (Uspenskaya et al., 2016). This technology enables to produce protein hydrolysate, which can be used as a stimulator of plant growth and development for cultivation of berries with increased phenol compounds. The vegetation period is decreased by 10–14 days for the climate conditions of Russian north-west regions. Further berries quick-freezing using fre-flo freezer allows to produce semi-products of high quality. Its application in confectionery with agar or starch embedding makes products competitive. (Garrido et al., 2016; Arfat et al., 2017).

The purpose of the work was to determine the optimum concentrations of treatment of garden plants by hydrolysate of meat processing by-product – beef split and possibilities in usage of fruit and berry fast-frozen products.

## MATERIALS AND METHODS

Canned beef split was used to receive the protein hydrolysate as the stimulator. Beef collagen tissue was preserved with calcium hydroxide, treated with hydrochloric acid to neutralize calcium hydroxide and then rinsed with running water. Then two-stage hydrolysis was performed. The first stage included water hydrolysis at 100 °C for 1.0–1.5 hours, decantation of liquid phase, homogenization of solid phase and mixing of both phases into suspension. At the second stage the suspension was hydrolysed with 0.012 – 0.250% hydrochloric acid solution under 130–150 °C and hydro modulus of 1: (1.5–2.0) with a pressure of 3 atm during 4–6 hours, followed by drying in the drying machine with counter-curved flows of inert support with 180 °C input and 95 °C output temperature (Kutsakova, 2004).

Amino acid composition of the modified protein products was determined by the method of ion exchange chromatography on the automatic amino acid Analyser (Japan). The research results are presented, and also the characteristics of collagen hydrolysate are given below. Dry matter content was determined to the constant weight by the drying method at a temperature 105 ± 0.5 ºC. Calcium ions content was determined by the method based on measuring the electromotive force of the element that consists of a reference electrode with a known potential value and a calcium-selective electrode, which potential is determined by concentration of calcium ions in the examinee solution. The electromotive force is fixed by the converter, the operation principle of which is based on the transformation of ion activity to electro moving force values (mV), which is linearly dependent on ion activity in the analysed solution and its temperature. The electrode system includes an auxiliary reference electrode (silver chloride) and a measured electrode – calcium-selective. The temperature compensator is also applied for measurements. Active acidity (pH) of the hydrolysate solution with a mass fraction of 1% was determined by the potentiometric method. The mass fraction of sodium chloride was determined by the method of protein substances precipitating by solution of nitric acid and titration of the chlorides in an acidic extract according to Folhard.

Wide research of treated plants (meadow and green grasses, berry, fruit crops) in the North-West region of Russia was conducted. Objects of research were treated once (at acrospire and blossoming stage) with water solutions of hydrolysate with 50 to 800 mg $l^{-1}$ concentration using atomizing nozzle. Studies connected with treatment of sweet cherry trees and black currant bushes are presented in this paper. Single-factor experiment was used. A step of varying the concentration of 200 mg $l^{-1}$ to establish the limiting, stationary and inhibitory area was established. The perennial bushes and fruit trees without stimulator treatment were the control group. The objects treatment was carried out in triplicate with the water solution the stimulator concentration of 200, 400, 600, 800 and 1000 mg $l^{-1}$. Leaves research was presented for better stimultor concentration for black currant bushes (600 mg $l^{-1}$) at vegetation period.

Concentration of chlorophyll and carotenoids were used to determine the level of photosynthesis activity and concentration of photosynthesis products. Chlorophylls and carotenoids concentration in leaves, phenolic compounds in fruit and berries were determined by spectrophotometric method. The aboveground mass in the blossoming and fructification phase was collected. Selection was made from the area of 30x2 m, allocating 5 platforms of 1x1 m for each species of plants. Leaf samples for the research were taken triply. The pigment content was determined in an acetone extract (100%

concentration) using a spectrophotometer, in cuvettes with a layer thickness of 10mm by the absorption centers on lengths of waves of 644, 662 and 440.5 nm for chlorophyll a, b and carotenoids (k) respectively. The pigment concentration was calculated by Wettstein equations:

$$C_a = 9.78 \times D_{662} - 0.99 \times D_{644};$$
$$C_b = 21.42 \times D_{644} - 0.99 \times D_{662};$$
$$C_{a+b} = 5.13 \times D_{662} + 20.43 \times D_{644};$$
$$C_k = 4.69 \times D_{440.5} - 0.268(C_{a+b}),$$

where: $C_{(a;\ b;\ a+b;\ k)}$ – pigment concentration, mg $l^{-1}$; D – optical density in the centre of pigment absorption on the set wavelength.

Pigment content was calculated by the equation:

$$X = (C_{(a;b;a+b;k)} \times V)/(P \times 1000),$$

where: X – pigment content in mg per 1g of crude batch weight; V – volume of pigment extraction, ml; P – leaf batch weight, g. (Shlyk, 1971).

Leaf juice drying was performed in the drying unit (Kutsakova, 2004) at an air inlet temperature of 140 °C and an outlet of 90 °C. An average sample weight was 0.3 kg. In dry leaf juice the following parameters of the product safety were determined: the quantitative content of toxic elements – lead, cadmium, copper, zinc, mercury, and arsenic – by the method of inversion voltammetry. The method is based on the determination of the mass concentrations of elements in the sample solution that are defined by the method of adding certified mixtures of the determined elements, which do not demand a calibration curve construction. Using this method the voltammogram cycles are recorded. Residual amount of organochlorine pesticides – aldrin, hexachlorane, DDT and its metabolites were detected by method of chromatography in a thin layer – by gas-liquid chromatography.

Fruit sampling was performed by the method of medium samples extraction that implies taking the samples from no less than 10 trees and bushes located on the diagonal sections. The average sample mass was 0.2 kg to study each option.

To study the parameters of fresh, chilled, frozen fruit as well as the ones at fruit refrigeration storage, the following methods were applied. To determine phenolic compounds from fruitan alcohol extract was obtained. (Katserikova, 1998) To determine the total content of phenolic compounds (phenol carbonic and oxycinnamic acids), 0.3 $cm^3$ of the Folin-Denis reagent was added to 1 $cm^3$ of alcohol extract. After stirring the mixture for 20 sec. 5 $cm^3$ of a 20% solution of $Na_2CO_3$ was added. After 30 sec. the optical density at the wavelength of 725 nm was measured. The number of phenolic compounds was calibrated according to the curve that was constructed according to chlorogenic acid. The calculation was performed by equation:

$$X = aVpm^{-1},$$

where: X – number of phenolic connections of mg per 100 of crude weight; a – content of chlorogenic acid according to the calibration curve, mg $cm^{-3}$; V – volume of alcohol extract, $cm^3$; p – extent of dilutions; m – mass of a hinge plate, g. (Katserikova, 1999).

Determining the sum of flavonols of the initial alcohol extract 2 $cm^3$ of a 2% solution of $AlCl_3$ and 6 $cm^3$ of a 5% solution of sodium acetate were added. To a control sample 2 $cm^3$ of $H_2O$ was added instead of 2 $cm^3$ of 2% solution of $AlCl_3$. If solutions became turbid, they were filtered. After 2.5 hours after the beginning of the reaction the optical density at a wavelength of 440 nm was measured. The content of the sum of flavonols (mg per 100g in terms of rutin) was found by equation (Ermakov & Arasenovich, 1987):

$$X = K(D - D_1)Vpm^{-1} \times 100,$$

where: K – recalculation coefficient on the calibration curve constructed on a routine (0.655); D – optical density of a test solution; $D_1$ – optical density of a control solution; V – volume of a spirit extract, $cm^3$; p – extent of dilutions; m – mass of a hinge plate of plant material, g (Katserikova, 1999).

To find the number of anthocyanins according to the calibration curve needs to define their optical density (Tanchev, 1980) in alcoholic extracts at a wavelength of 529 nm. The quantity of anthocyanins (mg per 100g) is found by the equation:

$$X = KDVm^{-1} \times 100,$$

where: K – coefficient calculated on a calibration curve; D – optical density of solution; V – volume of extract, $cm^3$; m – mass of a hinge plate, g (Durmishidze et al., 1981).

The content of the tannin-catechin complex was conducted by the titration method with $KMnO_4$ solution recalculating results by equation:

$$X = (a - b)m^{-1} \times 5.82 \times 100 \times 100 \times 10^{-1},$$

where: a and b – quantity of $KMnO_4$ of $cm^3$ for test and control titration; m – a hinge plate of a dye, g (Bokuchava et al., 1976).

The activity of peroxidase and polyphenol oxidase determination was performed by standard iodine solution titration methods. (Ermakov, 1987) The results of the studies are represented on the example of sweet cherry and black currant fruit. The trees were sprayed with protein hydrolysate water solutions in concentrations of 200, 400, 600 and 800 mg $l^{-1}$. The black currant bushes were treated by the solutions with the concentrations of 400, 600 and 800 mg $l^{-1}$. Then both control and test fruit samples were frozen by fluidization method.

Fruit with initial temperature of 19–22 °C were frozen in an industrial quick–freezing machine with directed fluidized layer. Raw material was continuously fed into the machine and subjected to jets of air cooled to temperature of -28–-35 °C. Air coolant speed was 2.5–2.9 m $s^{-1}$. Quick–freezer design features provided placing fruit uniformly throughout the apparatus. That allowed to freeze each berry from its surface to the centre while moving at constant rate along the apparatus. The processed product was held inside the machine. The product layer was regulated to be 5 to 8 cm by a restraining device. Maximum throughput of an experimental apparatus for quick-frozen berries production was 20 kg $h^{-1}$.

Statistical data processing was carried out by using the software package of Microsoft Word 2010, STATISTICA Application (Confidence interval was 0.95).

## RESULTS AND DISCUSSION

High quality protein ingredients (hydrolysates) used as the stimulator of plant growth and development (the stimulator, growth-promoting agents) consist of finely dispersed powder of white or light-yellow colour with dry solids weigh ratio as much as 95%. Weight fraction of $Ca^{2+}$ on dry product basis is as much as 0.14%. pH of the hydrolysate solution with a mass fraction of hydrolysate of 1% is 5–7, the mass fraction of sodium chloride is 4%. Essential active substances of hydrolysate are amino acids in following quantities (%): glycine – 9.01; proline – 6.56; alanine – 5.17; glutamine acid – 6.03; lysin – 3.36; leucine – 1.57; aspargine acid – 2.06; valine – 1.64; serine – 0.84; histidine –0.84; isoleucine – 0.45; arginine – 0.31; methionine – 0.03 and trace amounts of threonine.

Using the hydrolysate as the stimulator for cropping, the specific combination of amino acids, particularly α- and β-amylases, results in accelerated plant growth acceleration. In north-western region of Russia ripening of treated plants starts 10–14 days earlier compared to the control group. The stimulators of plant growth and development are used in hydrolysed form under carefully selected component concentration specific to a particular plant. If the concentration is less than that of an optimal solution, the lack of sufficient number of units of active ingredients prevents enzyme synthesis. If the concentration is greater than optimal the growth rate of the plants becomes impeded as a result of fermentation inhibition. (Berg & Gundersen, 2003; Burey et al., 2008; Roussos et al., 2009; Taştan et al., 2012; Rahman et al., 2013)

Chlorophyll is the main pigment responsible for leaves colour. Chlorophyll concentration in leaves can be used to judge about the level of photosynthetic activity and degree of plant saturation with its products. Aside from that chlorophyll is also a K provitamin. Due to the fact that black currant leaves can be used as raw material in pharmaceutical and cosmetic industry the research also included determination of content of *a* and *b* chlorophyll and carotenoids. Leaves were gathered from bushes both untreated (control group) and treated with the stimulator. The acquired data is provided in Table 1.

As seen from the data acquired, chlorophyll concentration in treated bushes is always higher during the vegetation period compared to the untreated bushes from the control group. Total chlorophyll content in bushes from the control group is at its peak during harvest time. Increased content of chlorophyll and carotenoids are observed in leaves of treated plants. This can be explained by the fact that amino acids participating in metabolic reactions cause protein synthesis, which result in increase in chlorophyll concentration. Thus, proper plant treatment with the stimulator increases its photosynthetic activity.

Such intensive chlorophyll synthesis during the most active period of the plant growth (fast growth period preceding blossoming) can be explained by high productivity of assimilating organs producing and providing high quantity of assimilates to the plant.

During blossoming and fruiting periods the significant depletion of leaf yellow pigment is observed. However, during the activation of bio production process, chlorophyll amplification and carotenoids biosynthesis occurred. It might be necessary

to supply activated process of organic substance formation of the vegetative parts of the plant with assimilates. Until the end of vegetation there was a trend of total chlorophyll content decreasing, which relates to the process of pigment suppression. The second stage of carotenoids biosynthesis activation is accompanied by the weakening of bio production process. Such dependence between the content of pigments in leaf tissue and growth function of plant development was previously observed. As for the ontogenetic rhythms of green pigment accumulation within leaf vegetation period, the proportion of its different forms is observed. At the time of the most intense chlorophyll biosynthesis ratio of *a*-chlorophyll to *b*-chlorophyll decreased. Obviously, *b*-chlorophyll is synthesized at higher rate during plant blooming preparation and intense growth phases.

**Table 1.** Measurement of pigment content in treated black currant leaves by stimulator at vegetation period

| Month of research, sample | Black currant content*, mg $g^{-1}$ | | | |
|---|---|---|---|---|
| | Chlorophyll *a* | Chlorophyll *b* | Sum of chlorophyll | Carotenoids |
| May, control sample | 1.062 ± 0.008 | 0.134 ± 0.007 | 1.196 ± 0.015 | 0.659 ± 0.007 |
| May, stimulated sample | 1.261 ± 0.007 | 0.244 ± 0.005 | 1.505 ± 0.012 | 0.767 ± 0.008 |
| June, control sample | 1.287 ± 0.006 | 0.438 ± 0.003 | 1.725 ± 0.009 | 0.559 ± 0.003 |
| June, stimulated sample | 1.328 ± 0.008 | 0.707 ± 0.004 | 2.035 ± 0.012 | 0.525 ± 0.005 |
| July, control sample | 1.509 ± 0.005 | 0.826 ± 0.008 | 2.335 ± 0.013 | 0.409 ± 0.008 |
| July, stimulated sample | 1.652 ± 0.007 | 1.032 ± 0.007 | 2.684 ± 0.014 | 0.397 ± 0.004 |
| August, control sample | 0.552 ± 0.004 | 0.269 ± 0.007 | 0.821 ± 0.011 | 0.363 ± 0.007 |
| August, stimulated sample | 0.582 ± 0.007 | 0.272 ± 0.005 | 0.854 ± 0.012 | 0.379 ± 0.008 |
| September, control sample | 0.028 ± 0.006 | 0.008 ± 0.006 | 0.036 ± 0.012 | 0.266 ± 0.004 |
| September, stimulated sample | 0.142 ± 0.008 | 0.017 ± 0.004 | 0.159 ± 0.012 | 0.261 ± 0.004 |

* – Confidence interval ≥ 0.95

Thus, seasonal dynamics of pigment accumulation in black currant leaves shows a trend specific to particular plant species. The range of chlorophyll and carotenoid oscillations and rate of their accumulation was narrower in depleted agrochemical background conditions (control group).

Dry powder with solubility of 98% was obtained from black currant leaves juice. The powder contained the following amounts of toxic elements (mg $kg^{-1}$): Pb (5.2), Cd (0.31), Cu (80.0), Zn (120.0), Hg (0.03), As (< 0.1); trace amounts of pesticides were (mg $kg^{-1}$): aldrin (not detected), heptachlor (not detected), hexachlorane (sum of isomers) (< 0.001), DDT and its metabolites (< 0.002).

The stone fruit and berry crops (plum, sweet cherry) treatment by protein hydrolysate accelerated the beginning of fructification. The 10–14 day increase in the rate of plant development made it possible to form a crop, 15–20% bigger in comparison with control group.

The stone fruit cultures treatment is performed by a stimulator water solution at a concentration of 600 mg $l^{-1}$ (stationary area) If concentrations are below optimal level (less than 600 mg $l^{-1}$) the activity of amylases is insufficient to obtain a higher yield of fruit (limiting area). When using concentrations exceeding the optimal level (more than 600 mg $l^{-1}$), the activity of amylase decreases slowing the rate of organogenesis stages (inhibitory area). Plant organogenesis is responsible for accelerating development, beginning of fruiting and achieving maximum productivity.

Experimentally established relationship between the concentrations of the stimulator (a protein hydrolysate), the content of polyphenol compounds (in particular anthocyanins responsible for staining stone fruit and berry crops, possessing P-vitamin activity and antioxidant properties) and the activity of oxidative enzymes-polyphenol oxidase (PFO) and peroxidase (PO). The corresponding relationships are shown in Figs 5 and 6.

At protein hydrolysate concentrations, less than 600 mg $l^{-1}$, the total number of amino acids supplied to plant cells with a stimulator and nitrogen fertilizers is insufficient for intensive activity and proteolytic and oxidative enzymes. Therefore, when using the stimulator concentrations below 600 mg $l^{-1}$, it is impossible to achieve neither an increase in the content of polyphenol compounds, in particular anthocyanins, nor an increase in yield.

At stimulator concentrations bigger than 600 mg $l^{-1}$, the plant has a significant number of amino acids for the synthesis of enzymes. As a result of high activity of proteolytic enzymes (α- and β-amylases), there is an active formation and outflow of nutrients to fruit, which lead to 15–20% increase in yield by. However, high activity of oxidizing PFO and PO enzymes causes a decrease in the content of polyphenolic compounds, including anthocyanins preventing the production of fruit with specified properties.

At optimal concentration of protein hydrolysate 600 mg $l^{-1}$, a stone fruit had the maximum content of anthocyanins.

Thus, treating stone fruit at optimal concentration, the application of a protein hydrolysate promotes the production of a high fruit yield with more intense colouring substances of fruit caused by the increased content of polyphenolic compounds, in particular anthocyanins.

An advantage of plants is their ability to effectively use amino acids, supplied by the stimulator in strictly defined development phases, established according to the stages of organogenesis. Hence, the treatment of stone fruit crops is performed during the blossoming phase. The results of the study allowed to develop a technique for the use of the stimulator for berry and stone fruit crops.

The main way of processing stone fruit crops is spraying by injectors. The flow rate of the working fluid depends on the quality of the spray (uniform and complete wetting of a surface of plants are crucial), weather conditions (the possibility of flushing the stimulator from the plants in case of rain should be considered), and the vegetative phase. Plants should be sprayed either in the morning or in the evening hours, so that the stimulator gradually penetrated into the plant with the most efficiency. The working solution is prepared immediately before the use and cannot be stored. This invention advantage is in the usage of the stimulator for fruit growth and development. The protein stimulator consists of polyphenolic connections, in particular anthocyanins having P-vitamin activity and antioxidant properties, to accelerate the beginning of fruiting by 10–14 days.

Moreover, double stimulation with a protein hydrolysate of 400 mg $l^{-1}$ is possible and positively affects the accumulation of biologically active agents of berry raw materials. Further research allowed to determine the optimum concentrations of the stimulator in a single treatment of plants. For sweet cherry, cherry, plums trees; gooseberries, black and red currant bushes water solution of stimulator with a concentration of 600 mg $l^{-1}$ is recommended.

In this work we have studied the effect of the stimulator concentration on the quality of grown berries and fruit in a single treatment of bushes and trees, for example, on the content of substances with P-vitamin activity. Fruit trees and bushes were processed in the flowering phase with the solutions of the stimulator in various concentrations.

All mentioned grades are zoned and recommended for the cultivation in the North-West region of Russia.

As the harvest of fruit and berry raw materials occur within 2–3 summer months in the North-West region of Russia, it is desirable to prolong the possibility of fruit consumption all year round. Therefore, it is necessary to develop a technology for their refrigeration processing and storage. For fruit processing with freezing, raw material collecting is recommended at the stage of semimature. By this time, the process of accumulation of reserve substances and ripening of seeds is basically completed, a fruit acquires a required size, appearance, consistence, colour and taste, so they are suitable for technical processing and transportation. Fruit collected at the stage of full maturity are recommended for fresh consumption as they are not suitable for processing.

It is necessary to analyse the changes in the content of biologically active substances in native products and in their refrigerated storage.

**The influence of the stimulator concentration on the content of phenolic compounds of fruit and berry raw materials.** The aim of the experiment was to select fruit and berry raw materials, most suitable for the production of confectionery products, sauces, and other products, including stone fruit and berry crops.

Due to the fact that the anthocyanins content in treated fruit and berry raw materials with the stimulator of 1,000 mg $l^{-1}$ concentration is practically equal to the parameter content in the control group, fruit and berries were treated with water solutions of the stimulator of 200, 400, 600 and 800 mg $l^{-1}$ concentrations. The stimulator concentration increasing (1,000 mg $l^{-1}$) does not stimulating action on plant facilities and deteriorate its chemical composition. Figs 1–6 show the changes in sweet cherry fruit depending on the concentration of the stimulator collected at the stage of semimature.

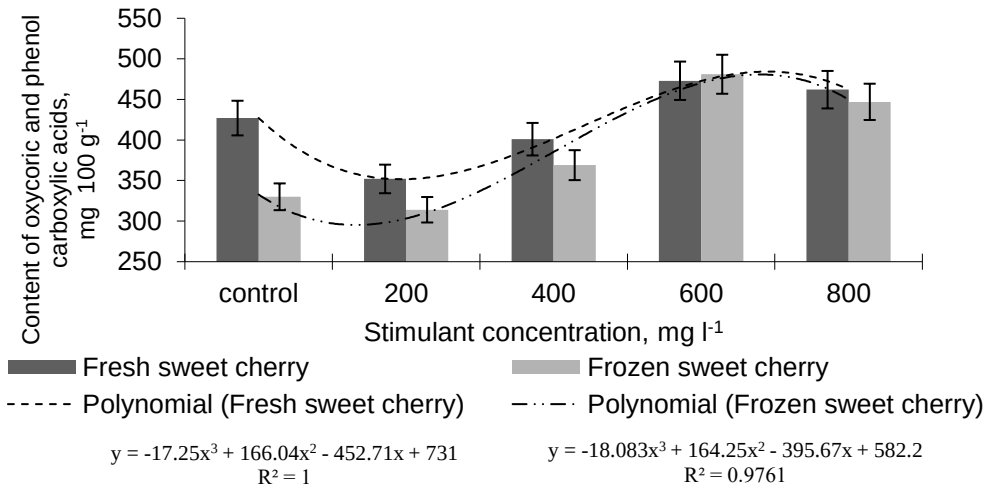



**Figure 1.** Dependence of the content of oxycoric and phenol carboxylic acids in sweet cherry fruit fresh and frozen on the concentration of protein hydrolysate.

As seen from the dependencies represented in Figs 1–6, the content of flavonols and anthocyanins in all experimental samples exceeds the values of the investigated parameters with respect to the control ones. In fruit, treated with a concentration of 200 mg $l^{-1}$, the highest content of flavonols was found (Fig. 2) with respect to other experimental samples, maximum polyphenol oxidase activity (Fig. 5) and peroxidase (Fig. 6) was observed. The low content of oxycoric and phenol carboxylic acids can be explained only by the fact that most of them are spent on the synthesis of flavonols and tannic-catechol complex (Fig. 3). This results in the incomplete maturation of the fetus.

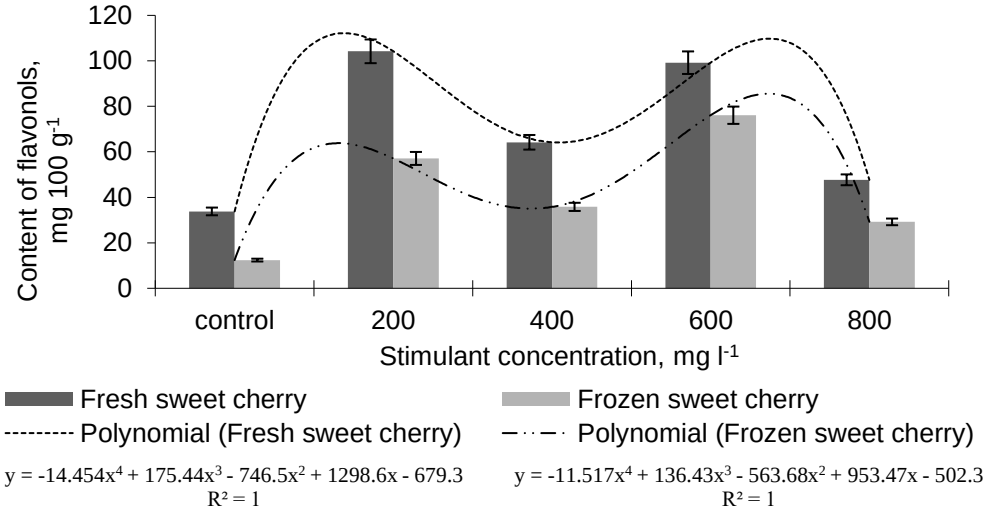



**Figure 2.** Dependence of the content of flavonols in sweet cherry fresh and frozen on the concentration of protein hydrolysate.

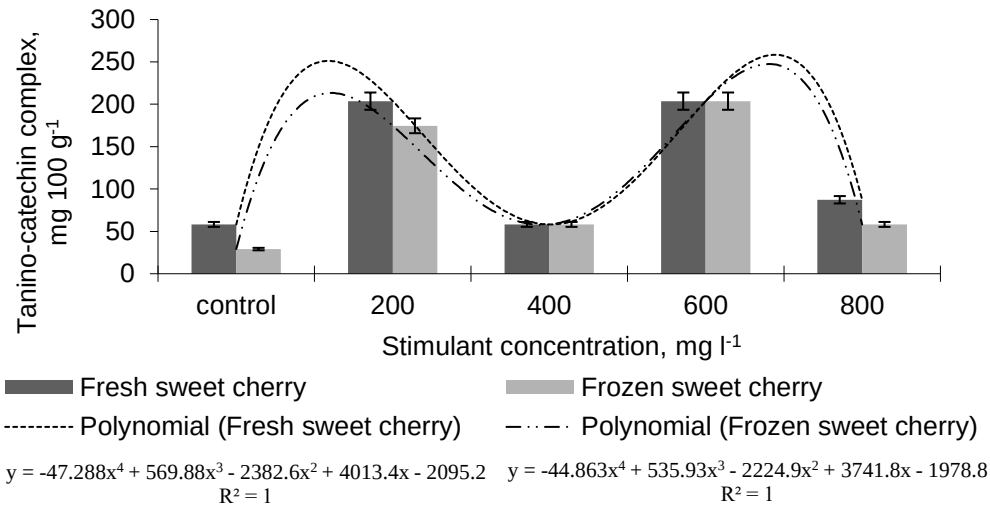



**Figure 3.** Dependence of the content of the tannic-catechol complex in sweet cherry fruit fresh and frozen from the concentration of protein hydrolysate.

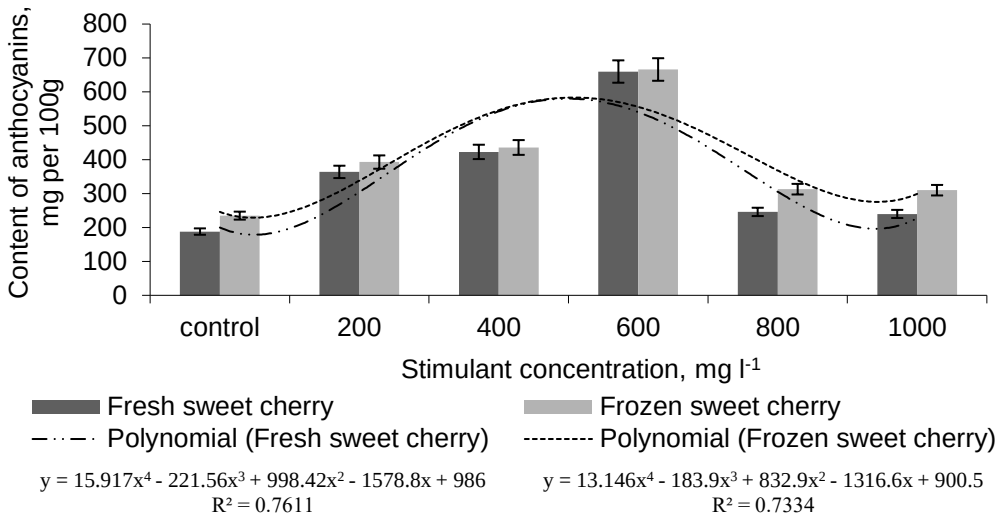



**Figure 4.** Dependence of the content of anthocyanins in sweet cherry fruit fresh and frozen on the concentration of protein hydrolysate.

The unfinished maturation process was also observed in fetuses that were treated with the stimulator of the concentration of 400 mg $l^{-1}$: the synthesis of phenolic compounds was not completed, their content was lower than in other samples. However, the activity of oxidizing enzymes (Figs 5 and 6) is close to zero, the content of the tannic-catechol complex is numerically equal to the content in the control sample and is 58.2 mg 100 $g^{-1}$.

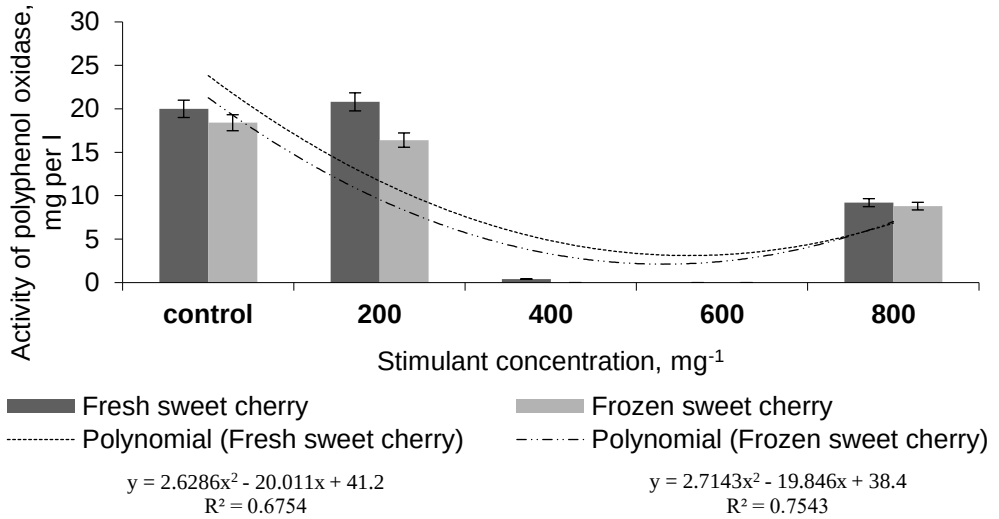



**Figure 5.** Change in the activity of polyphenol oxidase in fruit of sweet cherry fresh and frozen from the concentration of protein hydrolysate.

The highest content of anthocyanins (660 mg 100 $g^{-1}$, Fig. 4), oxycoric and phenol carboxylic acids (Fig. 1), as well as tannins (Fig. 3) was in sweet cherry samples treated with a stimulator concentration of 600 mg $l^{-1}$. At the same time, the activity of polyphenol oxidase (Fig. 5) and peroxidase (Fig. 6) is small. In other words, the concentration of the growth stimulant affects the rate of accumulation of phenolic compounds.

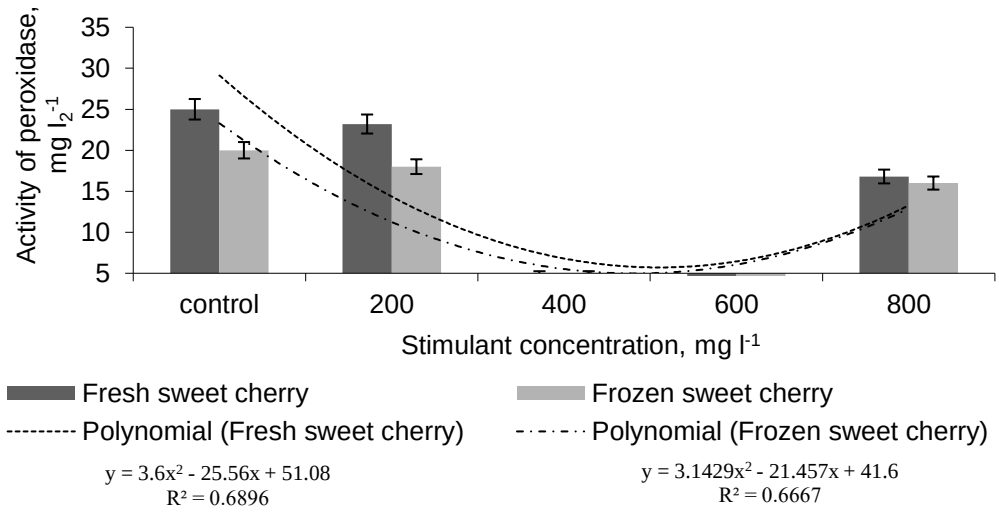



**Figure 6.** Change in the activity of peroxidase in fresh and frozen sweet cherry fruit from the concentration of protein hydrolysate.

It is possible to assume that the inhibition of enzymes is influenced by tannins; their content at the time of sweet cherry harvesting is sufficient for PFO and PO oppression. With further maturation, the activity of the enzymes increases and leads to the oxidation of various groups of phenolic compounds. Hence, the accumulation of phenolic substances with a lower molecular weight happen. With the concentration of the stimulator to 800 mg $l^{-1}$, no noticeable increase in phenolic connections is observed, their concentrations are close in value to the control sample.

Biosynthesis of phenolic compounds is conducted on the shikimate and acetate-malonate pathways. The predecessor of ring B is shikimic acid (or L-phenylalanine), and the predecessor of ring A is acetate (malonate). The sources of shikimic acid formation are the products of glycolytic decomposition of sugars and pentose phosphate cycle: phosphoenolpyruvic acid and erythrose-4-phosphate. The initial connection of the acetate-malonate pathway – acetyl-coenzyme A, which is formed either by oxidative decarboxylation of pyruvic acid, or in a thiokinase reaction containing a macroergicthioether bond. There are reasons to suppose that the $C_6$-$C_3$ fragment, which was formed by shikimat path, participates in the synthesis of flavonoids in the form of a corresponding ester with coenzyme A, performing the function of a matrix for the configure of activated acetate (malonate) residues. Chalcones formed during these reactions are the precursors of phenolic compounds. As known the hydroxylation of ring B occurs at earlier stages of biosynthesis, i.e. at the stage of cinnamic acids. Moreover,

it should be noted there is no enzyme that is capable to introduce of the third hydroxyl group into the ortho position to the two molecules of flavonoids already present in B rings. In the shikimic acid molecule 3,4,5-trioxygrouping already exists, and probably higher plants have a mechanism that is able to preserve this substitution during the biosynthesis of flavonoid compounds.

The dynamics of phenolic connections accumulation shows that the stimulated berries are active metabolites. The quantitative ratios of oxycoric and phenolic carboxylic acids, flavonols, anthocyanins, and tannic-catechol complex, as well as the activity of PFO and PO, which vary during plant vegetation, are most likely associated with the formation of either more oxidized or reduced forms of phenolic compounds.

Changes in phenol compounds in black currant berries at the stage of semimature intended for industrial processing (quick freezing) gathered on 20th of July (sample no. 1) and berries at the stage of full maturity, gathered on 25th of July (treated with the stimulator) and 2nd of August (the control group) (sample no. 2) are shown in Table 2. It should be noted that samples no. 2 were gathered at different time, because at the moment of harvesting both berries treated and berries untreated did not reach full maturity condition according to organoleptic estimation.

**Table 2.** Content of phenol compounds in black currant berries

| Conc. of stimulator, mg $l^{-1}$ | Polyphenolic compounds, mg 100 $g^{-1}$ | | | | | |
|---|---|---|---|---|---|---|
| | Flavonols | | Phenol compounds | | Anthocyanins | |
| | Sample no. 1 | Sample No. 2 | Sample no. 1 | Sample no. 2 | Sample no. 1 | Sample no. 2 |
| Control | 171.5 ± 0.3 | 211.3 ± 0.2 | 120.0 ± 0.4 | 120.9 ± 0.2 | 1.415 ± 0.002 | 1.860 ± 0.004 |
| 400 | 179.3 ± 0.2 | 231.1 ± 0.3 | 126.4 ± 0.2 | 136.1 ± 0.3 | 1.333 ± 0.005 | 2.368 ± 0.002 |
| 600 | 187.9 ± 0.4 | 258.6 ± 0.4 | 145.9 ± 0.3 | 154.6 ± 0.4 | 1.923 ± 0.003 | 2.602 ± 0.002 |
| 800 | 173.9 ± 0.3 | 228.6 ± 0.2 | 134.4 ± 0.4 | 146.4 ± 0.3 | 1.934 ± 0.004 | 2.573 ± 0.003 |

The data demonstrates the concentration of chlorogenic acid, quercetin and anthocyanin increase as the berries of black currant grew while being treated with the stimulator with concentrations of 400, 600 and 800 mg $l^{-1}$.

The greatest content of phenol compounds was found in berries treated with the stimulator with the concentration of 600 mg $l^{-1}$ gathered at both semimature and full maturity stages. Moreover, at the same concentration the lowest enzymatic activity was observed (Table 3). Relative to the control group samples, samples gathered at the stage of semimature showed a higher concentration of oxycinnamic and phenolic carboxylic acids by 21.6%, flavonols by 9.6%, anthocyanins by 35.9%. Samples gathered at the stage of full maturity also demonstrated increased concentrations of these components: the content of oxycinnamic and phenolic carboxylic acids was higher by 27.8%, flavonols by 22.4%, anthocyanins by 39.9%. As seen from the data provided, relatively to the control group samples the accumulation of phenol compounds is much more intense for berries gathered at the stage of full maturity rather than for the ones gathered at the stage of semimature. Treated berries reached the stage of full maturity 10 days earlier than the control samples and berries had a higher concentration of phenol compounds (Bushkov et al., 2016; Rodriguez-Furlán et al., 2016).

Thus, it can be concluded that the processing of storage fruit and berry crops grown with the use of the stimulator with the concentration of 600 mg $l^{-1}$ is advantageous.

The value of black currant berries as a source of P-active substances is usually determined by the high content of anthocyanins, represented by the derivatives of cyanidin and delphinidin: cyanidin-3-rutinoside, delphinidin-3-monoglycoside and delphinidin-3-rutinoside. During the vegetation period, the total content of colouring substances gradually increases, the periods of active growth of fruit and maturation being characterised by the most intensive accumulation. Quantitatively, in the most anthocyanins fruit and berries, colouring substances predominate over other flavonoids, especially flavonols.

**Table 3.** Changes in the activity of polyphenol oxidase (PPO) and peroxidase (PO) in black currant berries collected in stage of semimature

| Enzyme | Concentration of stimulator, mg $l^{-1}$ | | | |
|---|---|---|---|---|
| | control | 400 | 600 | 800 |
| Activity of PPO, mg $I_2^{-1}$ | 63.8 ± 0.4 | 55.6 ± 0.2 | 20.4 ± 0.2 | 23.2 ± 0.5 |
| Activity of PO, mg $I_2^{-1}$ | 30.5 ± 0.1 | 16.3 ± 0.3 | 1.0 ± 0.1 | 26.5 ± 0.4 |

The content of anthocyanins in blackcurrant berries collected at the stage of full maturity reaches 2602.9 mg 100 $g^{-1}$, and at the semimature stage – 1923.4 mg 100$g^{-1}$. Freezing is recommendable for berries collected at the technical stage. Maturity, since during the subsequent refrigeration a number of enzymatic reactions occur. These processes approximate a product properties to the stage of full maturity.

It should also be noted that a high content of phenolic compounds in all samples of black currant berries may be connected with weather conditions. So high temperatures (up to 30 °C) in the first half of the month had a positive effect on the accumulation of flavonols and phenols, while temperature decrease in the second half of July raise the content of anthocyanins.

Protein hydrolysate treated black currant berries, being fresh, frozen and 9 months of cold stored, had the content of phenols (oxycoric and phenol carboxylic acids) respectively: 145.9, 146.8, 197.2 mg 100$g^{-1}$; flavonols – 187.9, 188.1, 189.9 mg 100$g^{-1}$; anthocyans – 1923, 1972, 187 mg 100$g^{-1}$. In control samples, respectively, phenols – 120.0, 121.2, 193.4 mg 100$g^{-1}$; flavonols – 171.5, 173.8, 170.2 mg 100$g^{-1}$; anthocyanins 1415, 1482, 1375 mg 100$g^{-1}$.

In red currant berries, treated with stimulator in the concentration of 600 mg $l^{-1}$, had the maximum accumulation of essential substances – flavonols (162.8 mg 100$g^{-1}$), phenols (75.2 mg 100$g^{-1}$) and anthocyanins (235.6 mg 100$g^{-1}$).

In addition, the sugar-acid index in the samples treated with the stimulator is 600 mg $l^{-1}$ higher in terms of the other samples considered, it affects their taste characteristics.

Thus, the processing of fruit and berry raw materials grown using the stimulator at a concentration of 600 mg $l^{-1}$ seems promising and allows obtaining raw materials with a high content of essential substances not only for its direct consumption, but also for the subsequent freezing.

**Freezing effect on phenolic compounds of fruit and berry raw materials and on their changes during subsequent refrigerated storage.** The quality preservation of fruit and berries for a long time is possible with the creation of modern technologies of rapid freezing and plants that ensure high speed of the freezing process.

To research the freezing effect on fruit and berry raw materials grown with the stimulator treatment, studies have been performed on the biochemical composition of raw materials during the freezing and in the process of refrigerated storage in a frozen state.

As sweet cherry fruit treated with the stimulator have the highest content of oxycoric and phenolic carboxylic acids, flavonols, anthocyanins and tannin catechol complex, they were used to examine the influence of low temperatures on phenolic compounds.

When frozen in relation to the content in the native berry, the content of phenols is increased by 2% (Fig. 7), the content of flavonols reduced by 23% (Fig. 8) and the content of anthocyanins increases by 1% (Fig. 9).

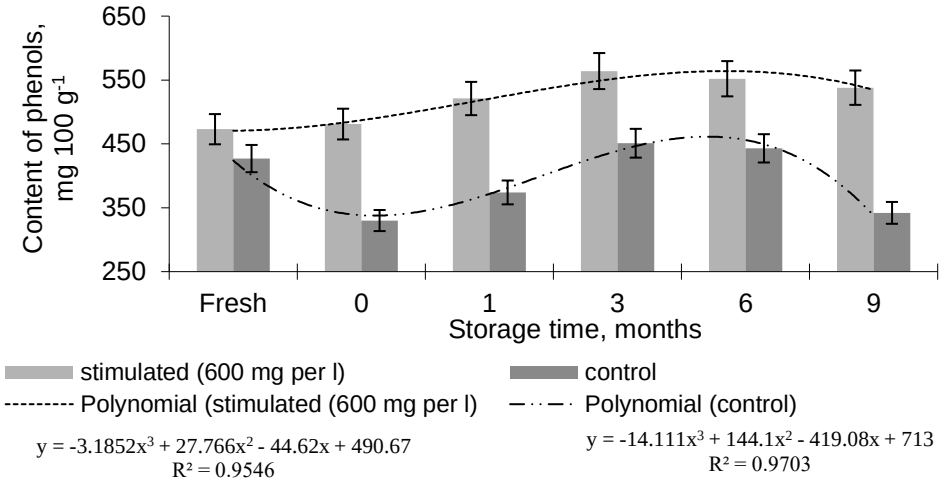



**Figure 7.** Dependence of the content of oxycoric and phenol carboxylic acids in sweet cherry fruit from the duration of storage in the frozen state.

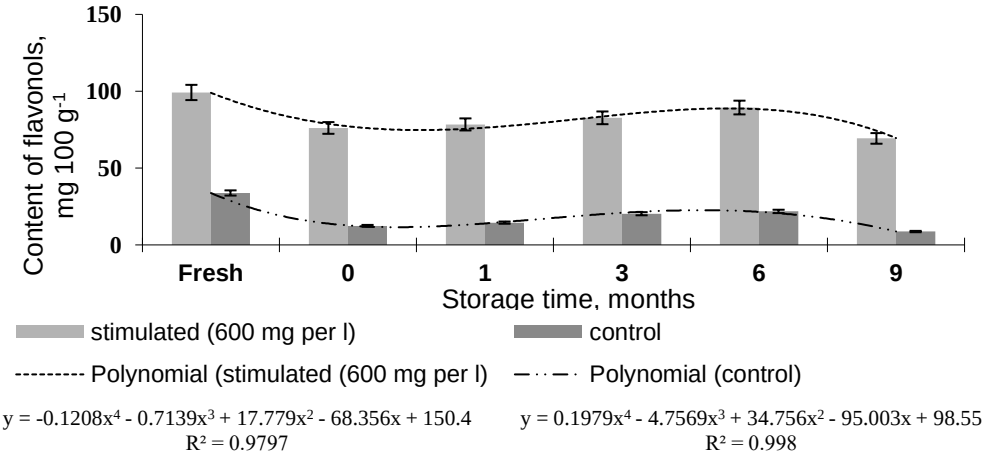



**Figure 8.** Dependence of the content of flavonols in sweet cherry fruit from the duration of storage in the frozen state.

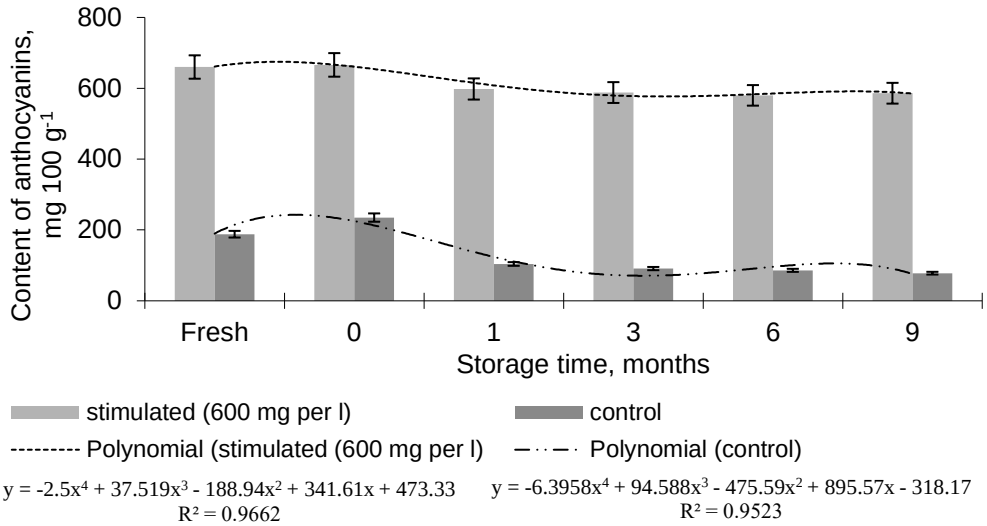



**Figure 9.** Dependence of the content of anthocyanins in sweet cherry fruit on the duration of storage in the frozen state.

To study the content of oxycinnamic and phenolic carboxylic acids (Fig. 9), a technique to determine these components only in a free, but not in a bound state was applied. Basically, the oxycoric and phenolic carboxylic acids of the fruit are either esterified or glycosidase. In the case of fruit refrigeration, the decomposition of oxycoric and phenol carboxylic acids with the elimination of sugar residues, the destruction of ester bonds causing the appearance of acids in a free form, and the accumulation due to the destruction of flavonoids of complex structures (anthocyanins, flavonols) to simpler compounds occur in fruit.

During the refrigeration the content of oxycoric and phenol carboxylic acids in sweet cherry increased by 12% comparing with the content obtained immediately after the freezing.

In fruit the flavonols (Fig. 8) as glycosides and aglycone forms in a smaller quantity are found. The technique used allows to determine only the aglycone forms of flavonols. During the freezing and refrigeration there is the decomposition of flavonols with the formation of simple precursors (oxycoric and phenolic carboxylic acids), and the conversion the flavonols glycosidic form into aglyconic form occurs. Due to this fact the content of flavonols after 6 months refrigeration was increased, however, the initial fresh fruit content was not reached. The content of flavonols in refrigerated sweet cherry decreased comparing with the content obtained immediately after the freezing by 9%.

As known not all moisture turns into ice in the process of freezing. A small part of it does not freeze providing enzymatic activity oxidoreductase and hydrolase primarily.

Probably, air oxygen and enzymatic activity cause the oxidation and decomposition of condensed tannins to simpler precursors – catechins and leucoanthocyanins.

The oxidative enzymes decomposition to simpler components (oxycoric and phenolic carboxylic acids), resulted in decreasing content of authocyanins in the course of refrigeration by 11% comparing with the content obtained immediately after freezing (Fig. 9).

There is a drop in the amount of biologically active substance in the fruit during freezing and further refrigeration. But the usage of the stimulator makes it possible to obtain a higher-quality product for confectionery, sauces, etc.

The biochemical composition of berries and fruit was measured with a fivefold. Confidence interval was 0.95 and Student's *t* test coefficient was 2.7764 (as fivefold measurement). The statistical part of the work was calculated for stimulated black currant leaves and berries with a concentration of 600 ml $l^{-1}$ collected in July (Table 4).

**Table 4.** Describing statistics for stimulated black currant leaves and berries with a concentration of 600 ml $l^{-1}$ collected in July

| | Standard deviations | | | Statistical significance | |
|---|---|---|---|---|---|
| | Arithmetical average | Variance | Standard deviation | Absolute error | Comparative error, %. |
| Chlorophyll *a* | 1.652 | $3.32\ 10^{-5}$ | 0.00576 | 0.007 | 0.43 |
| Chlorophyll *b* | 1.032 | $3.25\ 10^{-5}$ | 0.00570 | 0.007 | 0.69 |
| Carotenoids | 0.397 | $1.00\ 10^{-5}$ | 0.00316 | 0.004 | 0.99 |
| Flavonols | 187.9 | $8.50\ 10^{-2}$ | 0.292 | 0.362 | 0.19 |
| Phenol compounds | 145.9 | $6.50\ 10^{-2}$ | 0.255 | 0.317 | 0.22 |
| Anthocyanins | 1.923 | $0.65\ 10^{-5}$ | 0.00255 | 0.003 | 0.16 |
| Activity of PPO | 20.4 | $2.3\ 10^{-2}$ | 0.152 | 0.188 | 0.92 |
| Activity of PO | 1.0 | $10^{-2}$ | 0.1 | 0.124 | 12.42 |

## CONCLUSIONS

The improvement of technologies for the complex processing of by-products of collagen-containing raw materials new food products with the specified composition and properties is importance in meat, poultry, fish processing industries, in various sectors of canning (thermal and refrigeration) production, fat and oil production, confectionery production and others.

The semi-product of beef cattle derma collagen tissue is used in pharmacy and hospital line of products as well as in feedstuff. It is used as raw material to produce absorbable wound healing collagen haemostatic sponges, absorbable collagen burn sponges, absorbable collagen haemostatic sponges and finds its use in haemostatic and wound healing preparation and gel wound dressing. In food industry this semi-product is used to produce natural shells for a large variety of sausages. The usage of by-products of animal origin for the development of new technologies producing high-quality protein ingredients for various purposes is also possible. Agriculture in severe weather conditions demands an innovative stimulator of growth and development of plants to provide ample harvests. We have found that the stimulator treatment for sweet cherry trees (and other stone fruit), black (white, red) currant and gooseberry bushes the solution with the concentration of 600 mg $l^{-1}$ is used respectively. The stimulator can also be combined with mineral fertilizers, traditionally used in agriculture. Technologies for the application of hydrolysates – protein growth stimulators and plant development for berry, fruit and green crops, as well as meadow grasses have been developed.

The terms and concentrations of the stimulator have been determined. The stimulation effect on the properties of plant products, including the processes of refrigeration and subsequent cold storage, is established. In the conditions of the North-

West region of Russia, the fruit and berry raw materials treated ripen 10 – 14 days faster. Semimature black currant berries intended for further refrigeration treatment, have a higher content of phenolic compounds comparing with the one registered in the control group: oxicoric and phenol carboxylic acids by 21.6%, flavonols – 9.6%, anthocyanins – 35.9% and other essential substances: vitamin C – 12.4%, monosaccharides – 15.5%, sucrose – 20.0%, sugar index – 60.1%, with an increase in yield by 20–25%, and the timing maturing reduced in 6 – 10 days. It is important in terms of industrial production of berry crops in the North-West region of Russia. The stimulator treatment with the concentrations makes it possible to obtain raw materials with a high content of phenolic compounds. The further usage of such raw materials, that have an increased content of phenolic compounds, can be recommended for the creation various products for functional nutrition.

The use and creation of new technologies of quick-frozen berries is challenging. The products based on sol-gel matrices are also relevant to light, cosmetic, medical and other industries. The work prospects further development of new confection involving a sol-gel matrix. The product promises to be sustainable during cold storage and in high-temperature baking.

ACKNOWLEDGEMENTS. The authors are sincerely grateful to Bogomolov V.V., PhD, Head of Department, Federal State Budget Institution 'Leningrad Interregional Veterinary Laboratory' and Serajutdinova LD, Deputy General Director, State Centre 'Test – St. Peterburg' for the assistance in the determination of amino acid content and safety performance of the products.

## REFERENCES

Arfat, Y.A., Ahmed, J., Jacob, H. 2017. Preparation and characterization of agar-based nanocomposite films reinforced with bimetallic (Ag-Cu) alloy nanoparticles. *Carbohydrate Polymers* **155**, 382–390.

Berg, T.C., Gundersen, L.L. 2003. Synthesis of cytokininanalogs as potential plant growth stimulators. *In: 19th International Congress on Heterocyclic Chemistry*pp. 121.

Bokuchava, M.A., Pruidze, G.N., Ul'yanova, M.S. 1976. Biochemistry of production of vegetable dyes.*Tbilisi, Metsniereba publ*. 97 pp. (in Russian).

Burey, P., Bhandari, B.R., Howes, T., Gidley, M.J. 2008. Hydrocolloid gel particles: Formation, characterization, and application. *Critical Reviews in Food Science and Nutrition* **48**(5), 361–377.

Bushkov, N.A., Veselov, M.S., Chuprov-Netochin, R.N., Marusich, E.I., Majouga, A.G., Volynchuk, P.B., Shumilina, D.V., Leonov, S.V., Ivanenkov, Y.A. 2016. Computational insight into the chemical space of plant growth regulators. *Phytochemistry* **122**, 254–264.

Durmishidze, S.V., Shalashvili, A.G., Mzhavanadze, V.V. 1981. Flavonoids and oxycoric acids of some representatives of the wild flora of Georgia.*Tbilisi, Metsniereba publ*. 197 pp. (in Russian).

Ermakov, A.I., Arasenovich, V.V. 1987. Methods of biochemical study of plants.*Leningrad, Agropromizdat publ*. 430 pp. (in Russian).

Garrido, T., Etxabide, A., Guerrero, P., De la Caba. K. 2016. Characterization of agar/soy protein biocomposite films: Effect of agar on the extruded pellets and compression molded films. *Carbohydrate Polymers* **151**, 408–416.

Katserikova, N. V. 1998. Natural pigments as food additives.*Food indust*ry 4, 19. (in Russian).

Katserikova, N.V., Pozdnyakovskiy, V.M. 1999. Natural food colorings. *Novosibirsk, Ekor publ*. 58 pp. (in Russian).

Kutsakova, V.E. 2004. Drying of liquid and pasty products in a modified spouted bed of inert particles. *Drying Technology* **22**(10), 2343–2350.

Kutsakova, V.E., Frolov, S.V., Kremenevskaya, M.I., Marchenko, V.I. 2013. Pat. 2533037 RF: MPK C05 F 1/00, A 01 N 33/00. A method for producing a protein stimulator of plant growth and development / – № 201334879/13; appl. 24.08.13; publ. 20.11.2013. – Bull. № 32.6.

Rahman, M.M., Kabir, S., Rashid, T.U., Nesa, B., Nasrin, R., Haque, P., Khan, M.A. 2013. Effect of γ-irradiation on the thermomechanical and morphological properties of chitosan obtained from prawn shell: Evaluation of potential for irradiated chitosan as plant growth stimulator for Malabar spinach. *Radiation Physics and Chemistry* **82**, 112–118.

Roussos, P.A., Denaxa, N-K., Damvakaris T. 2009.Strawberry fruit quality attributes after application of plant growth stimulating compounds. *Scientia Horticulturae* **119**(2), 138–146.

Rodriguez-Furlán, C., Miranda, G., Reggiardo, M., Hicks, G.R., Norambuena, L. 2016. High throughput selection of novel plant growth regulators: Assessing the translatability of small bioactive molecules from arabidopsis to crops. *Plant Science* **245**, 50–60.

Shlyk, A.A. 1971. Determination of chlorophylls and carotinoids in green leaves extracts.*Biochemical methods in plant physiology. –M.: Nauka.* pp. 154–170 (in Russian).

Taştan, B.E., Duygu, E., Atakol, O., Dönmez, G. 2012. $SO_2$ and $NO_2$ tolerance of microalgae with the help of some growth stimulators. *Energy Conversion and Management* **64**, 28–34.

Tanchev, S.S. 1980. Anthocyanins in fruits and vegetables.*Moscow, Food industry publ*. 304 pp. (in Russian).

Uspenskaya, M.V., Kremenevskaya M.I., Volkova K.V., Sosnina O.A. 2016. Aquaculture: problems and modern perspective on topical solution. *Agronomy Research* **14**, 604–613.

*Agronomy Research* **15**(S2), 1348–1357, 2017

# A study of factors which influence mould spoilage in flat (sourdough) bread

L. Kuznetsova and O. Savkina*

Institute of Refrigeration and Biotechnologies, ITMO University, Lomonosova Street 9, 191002 St Petersburg, Russia;
*Correspondence: 1103savkina@mail.ru

**Abstract.** Bakery products are an excellent substrate for the development of microbial spoilage, especially mould spoilage and lime disease (otherwise known as chalk disease), because they have high levels of water activity $a_w$ = 0.94–0.97 and pH 5.5–6.0. Sliced bread in its packaging is highly susceptible to moulds and lime disease during storage. The aim of this study was to investigate the effects shown by the microbial contamination of flour quality, and the type of sourdough and organic acid, especially acetic acid, on mould spoilage in wheat and rye wheat bread. Microbial contaminations were studied in two batches of wheat flour and three batches of rye flour which had been manufactured in Belarus and Russia and in sourdough bread which had been produced using this flour. Investigated here was the impact of the quality and type of sourdough with various starter cultures of micro-organisms and the impact of the content of organic acid, especially acetic acid, on mould spoilage in wheat and rye wheat bread. The content of organic acids, including acetic acid, in different types of sourdough which has been prepared using different starter cultures and in different kinds of sourdough bread which have been studied using liquid chromatography. It was found that, in spite of the presence in flour of spore-forming bacteria, yeasts, and fungi, microbial contamination of the finished product immediately after baking was absent. It was proven that the use of starter cultures and sourdough can slow down or prevent entirely the microbial spoilage of bread. It was found that the content of acetic acid which had been accumulated during the fermentation of various types of sourdough served to effect the presence of mould spoilage on sourdough bread.

**Key words:** sourdough, bread, microbial contamination, mould, microbial spoilage

## INTRODUCTION

In Russia, Belarus, and Ukraine in recent years, a large volume of bread has been sliced, packaged, and wrapped. This is the precipitating in the microbial spoilage of bread due to the presence of bacteria (known as rope or ropy disease), and yeast (lime disease) or fungi (moulds). Packing and wrapping prevents the loss of moisture from the cut surfaces of the bread slices, allowing a humid atmosphere to form around the loaf. This provides a moist surface for moulds and yeasts on which to grow. Additionally, the bread has a relatively high moisture content and water activity ($a_w$ = 0.94–0.97) and a pH around 5.5–6.0. These factors makes the bread an excellent substrate for the development of microbial spoilage, especially when caused by mould and yeast (de Blackburn, 2008; Cauvain, 2012).

At the same time there is no microbiological safety standard in place for bakery products in these countries, except for bakery products which are produced with a filling (such as pies). Standards which govern the content of spore-forming bacteria and moulds in grain and grain products are not in place, both in Russia and in other countries. The content of spore-forming bacteria in the wheat flour was determined by the minimum time required for the development of ropy disease in bread. There are also standards in place to control the content of mycotoxins in the flour, but not for mould. Therefore, any study of the effects of microbial contamination of flour on the microbiological safety of bread is of interest.

Mould contamination of bread occurs mainly during transportation, cooling, cutting, and packaging operations. The bread is infected by direct contact with contaminated objects (transportation and packaging tools, hands, or clothing), or through the air. The degree of microbiological contamination in the bread reveals the sanitary conditions of the bakery in question. Contamination of a businesses premises and equipment leads to the secondary contamination of raw materials, semi-finished products, finished products, and packaging, and encourages the development of microbial spoilage of bread (De Blackburn, 2008; Cauvain, 2012).

The development of tools and effective methods which will help to improve microbial safety and the storage stability of bakery products is a very real problem, one which includes a number of questions. In recent years, the bio preservation of bread has gained increasing interest thanks to rising consumer demand. Lactic acid bacteria as bio-preservation organisms are of particular interest. Studies generate a great deal of interest where they are related to the use of substances of a microbial origin which are produced by the fermentation microflora of dough and sourdough as a protective barrier against the microbial spoilage of bakery products. Lactic acid bacteria are able to produce different kind of bioactive molecules, such as organic acids, fatty acids, hydrogen peroxide, and bacteriocins. For example, the acetic acid which is formed in the fermentation process can have an inhibitory effect on the development of spore-forming bacteria and mould (Clarke et al., 2002; De Blackburn, 2008; Cauvain, 2012).

Most of the baking companies which operate in Russia, Belarus, and Ukraine work with rye and wheat sourdough and starter cultures of lactic acid bacteria and yeasts from the collection of the St Petersburg branch of the State Research Institute of the Baking Industry. Regarding this, the study of the influence of Russian sourdough with starter cultures on the mould spoilage of bread is of interest.

The aim of this study was to investigate the influence of the microbial contamination of flour, the quality and type of sourdough, and the content of organic acids, especially acetic acid, which is formed during the fermentation in sourdough on the mould spoilage of wheat bread and rye wheat bread.

## MATERIALS AND METHODS

### Starter cultures and sourdough

Used in this study were two types of rye sourdough with humidity levels of 50% and 68%, and two types of wheat sourdough with humidity levels of 42% and 65%. The sourdough was prepared in accordance with the official instructions for Russian baking companies with the use of micro-organism starter cultures from the collection of the St Petersburg branch of the State Research Institute of the Baking Industry (Kosovan, 2008).

Lactic acid and yeast bacteria starter cultures have been widely used for many years as symbiotic compositions in bakery companies in Russia (Afanasjeva, 2003; Kosovan, 2008).

The following starter microbial composition and sourdough were used:

Three strains of lactic acid bacteria - L *brevis 5, L brevis 78,* and *L plantarum 63* - and one strain of yeast - *C milleri* - were used for the dense rye sourdough with a humidity level of 50% (Kosovan, 2008).

Four strains of lactic acid bacteria - *L brevis 1, L plantarum 30, L casei 26,* and *L fermentum 34* - were used for the yeast-free liquid rye sourdough with a humidity level of 68%, and for liquid wheat sourdough with a humidity level of 62%. Three strains of lactic acid bacteria - *L brevis 8, L brevis 27,* and *Lplantarum* 6 - and two strains of yeasts - *S cerevisiae 90* and *S minor 7* - were used for dense wheat sourdough (Kosovan, 2008). The technological parameters for the various types of sourdough are shown in Table 1.

**Table 1.** Technological parameters of different types of sourdough

| Biotechnological indicators | Dense rye sourdough | Yeast-free liquid rye sourdough | Dense wheat sourdough | Yeast-free liquid wheat sourdough |
|---|---|---|---|---|
| Mass proportion of moisture, % | 50.0 | 68.0 | 42.0 | 62.0 |
| Temperature, °C | 30.0 | 40.0 | 20.0 | 39.0 |
| The time of fermentation, h | 6-00 | 19-00 | 24-00 | 2-00 |

**Flour**

Two batches of wheat flour and three batches of rye flour were used, all of which were manufactured in Belarus and Russia.

**Bread preparation**

The formulations for the rye wheat bread, 'Darnitsky', are presented in Table 2. A proportion of the rye flour was replaced with flour which followed the sourdough composition in accordance with the existing instructions (Kosovan, 2008*)*. A total of 25% of the quantity of rye flour in the recipe was replaced by dense rye sourdough with a humidity level of 50%, and 20% of the quantity of rye flour in the recipe was replaced by yeast-free liquid rye sourdough with a humidity level of 68%.

**Table 2.** The formulations of rye-wheat bread 'Darnitsky'

| Raw materials, % | Bread 'Darnitsky' | | | |
|---|---|---|---|---|
| | Dense rye sourdough | Yeast-free liquid rye sourdough | | |
| Rye flour | 35 | 40 | 35 | 30 |
| Quantity of rye flour in sourdough | 25 | 20 | 25 | 30 |
| Wheat flour | 40 | 40 | 40 | 40 |
| Total flour | 100 | 100 | 100 | 100 |
| Sourdough | 40 | 49 | 61 | 73 |
| Yeast | 0.5 | 0.5 | 0.5 | 0.5 |
| Salt | 1.4 | 1.4 | 1.4 | 1.4 |
| Water | until dough humidity of 43% | | | |

The required quantity of sourdough was mixed with the rest of the flour in the recipe, along with the yeast, salt, and water, until the dough achieved a humidity level of 43%. After mixing, the dough samples were shaped into 400g loaves, placed in aluminium pans so that a moisture content test of 43% could be achieved, and leavened at 30 °C until the volume was twice that of the initial volume. The leavened dough samples were cooked in an oven at 210 °C for eighteen minutes.

The formulations for the wheat bread are presented in Table 3. A proportion of the wheat flour was replaced by flour which followed the sourdough composition in accordance with the existing instructions. A total of 10% of the quantity of wheat flour in the recipe was replaced by dense wheat sourdough with a humidity level of 42%, and 5% of the quantity of wheat flour in the recipe was replaced by yeast-free liquid wheat sourdough with a humidity level of 62%.

**Table 3.** The formulations of wheat bread

| Raw materials, % | Bread 'Darnitsky' | |
|---|---|---|
| | Dense wheat sourdough | Yeast-free liquid wheat sourdough |
| Wheat flour | 90 | 95 |
| Quantity of rye flour in sourdough | 10 | 5 |
| Wheat flour | 100 | 100 |
| Total flour | 15 | 12 |
| Sourdough | 0.5 | 0.5 |
| Yeast | 1.4 | 1.4 |
| Salt | until dough humidity of 47.5% | |

The required quantity of sourdough was mixed with the rest of the wheat flour in the recipe, along with yeast, salt, and water, until the dough achieved a humidity level of 47.5%. After mixing, the dough samples were shaped into roughly 400g loaves, placed in aluminium pans, and leavened at 30 °C until the volume was twice that of the initial volume. The leavened dough samples were cooked in an oven at 200 °C for eighteen minutes.

**Flour quality assessment**

An assessment was carried out on the quality of the flour by analysing the following properties: the mass proportion of moisture in the flour was determined by drying it at a temperature of 130 °C for a period of forty minutes, while the mass proportion of ash was determined by burning flour in a muffle furnace at a temperature of between 600 °C and 900 °C until complete ashing had taken place with subsequent a determination being made of the non-combustible residue; the 'Falling' value for flour was determined by using the Hagberg-Perten method (ICC Standard No 107/1 (1995)), gluten content in wheat flour was determined by the complete sifting of gluten from 25 g of flour, and weighing and acidity were determined by titration, using a 0.1 n. solution of NaOH.

**Sourdough and dough assessments**

An assessment was carried out on the quality of the sourdough and dough by making use of the following properties: the mass proportion of moisture of the flour was determined by drying it at a temperature of 130 °C for a period of forty minutes, while acidity was determined by titration, using a 0.1 n. solution of NaOH (State Standard of

the Russian Federation, 1996). The lifting capacity was determined by the rate at which it rose in a glass of water at a temperature of 32 °C for a 10g mass of dough shaped into a ball and with a humidity level of 45%. The increase in volume was calculated by the ratio between the final volume and the initial volume multiplied by 100%. The content of volatile acids was determined by neutralising the evaporated volatile acid using a 0.1 n. solution of NaOH. The alcohol content was determined by using the iodometric method which is based on the quantity of sodium thiosulfate spent in titration. The content of acetic and lactic acid in various types of sourdough which were prepared with different microbial starter cultures were studied using liquid chromatography.

**Baked bread assessment**

An assessment was carried out on bread and sourdough quality levels in relation to the following properties: the mass proportion of moisture in the flour was determined by drying it at a temperature of 130 °C for a period of forty minutes, while acidity was determined by titration, using a 0.1 n. solution of NaOH (State Standard of the Russian Federation, 1996).

The content of acetic and lactic acid in leavened bread was studied using liquid chromatography.

**Determining the microbial contamination of flour**

Microbial contamination of wheat and rye flour were studied. A study of the microflora of flour was carried out in the following way: 10 g of flour was added to 100 ml of sterile water and diluted to between $10^{-1}$–$10^{-6}$. From each dilution a 0.1 ml suspension was added to the surface of the meat-peptone agar in a Petri dish, and 1 ml of the suspension was introduced into a Petri dish and poured on top of malt agar which contained a total of 8% of dry solids.

**Determining the microbial contamination of baked bread**

Microbial contamination of wheat and rye bread were studied. The bread was prepared for microbiological analysis in the following way: immediately after baking in the opening of the oven, the loaves were packed into sterile paper, placed in a sterile room, and cooled to a temperature of between 25–28 °C. After cooling, the bread was cut in sterile conditions. A total of 10g of bread was added to 100ml of sterile water and diluted to between $10^{-1}$–$10^{-6}$. From each dilution a 0.1 ml suspension was added to the surface of the meat-peptone agar in a Petri dish, and 1ml of the suspension was introduced into a Petri dish and poured on top of malt agar which contained a total of 8% of dry solids.

**Determining the effect of the technology behind bread-making on the rate of appearance of mould spoilage**

The impact of the type and quality of sourdough upon various micro-organism starter cultures and the impact of the content of acetic acid on mould disease in bread were investigated. Descriptions of the variations in the technological parameters of sourdough are represented in Table 1. In order to determine the effect of the technology behind bread-making with different types of sourdough on the rate of the appearance of mould spoilage, model experiments were carried out by contaminating sterile slices of a pure culture of the mould, *Penicillium chrysogenum*. Immediately after baking in the

oven opening, the loaves were packed into sterile paper, placed in a sterile room, and cooled to a temperature of between 25–28 °C. After cooling the bread was cut in a sterile environment, with slices being taken at a size of 3.5×6.5 cm and at a thickness of 0.3–0.4 cm. The slices were placed in sterile Petri dishes.

An aqueous suspension of a pure culture of the mould, *Penicillium chrysogenum*, was prepared for the infection of slices of bread. The biomaterial of *Penicillium chrysogenum* was transferred from a tube containing a pure culture of mould grown on malt agar to 1ml of sterile water using 'Tween-80' and was thoroughly suspended. The suspension was inoculated into each slice of bread in five shots using a microbiological needle. Petri dishes with infected slices were incubated at a temperature of 25 ± 1 °C until the first signs appeared of a growth of mould colonies. *Penicillium chrysogenum* was used because the *Penicillium* species are by far the most common for bread (Legan, 1993; Lund et al., 1996).

In addition, a loaf of bread in its packaging after cooling to between 25–28 °C was placed in the storage at a temperature of 25 ± 1 °C until the first signs of the growth of mould colonies.

**Statistical analysis of the data**

All of the experiments were carried out a total of five times; data was processed by using methods for mathematical statistics at a theoretical frequency of 0.95.

## RESULTS AND DISCUSSION

In order to determine the role of the microbial contamination of flour in the microbial spoilage of wheat and rye wheat bread, five batches of flour were studied which had been developed at mills in Russia and Belarus. Spore-forming and other bacteria, yeasts, and moulds, the number of which varied widely, were identified in the flour (Table 4).

**Table 4.** Microflora of flour

| Parameters | Wheat flour | | Rye flour | | |
|---|---|---|---|---|---|
| | Russia | | Belarus | | Russia |
| | Michurinsk | Barnaul | Polotsk | Baranovichi | S-Petersburg |
| Mass proportion of moisture, % | 14.3 | 11.9 | 12.6 | 13.0 | 13.1 |
| Mass proportion of ash, % | - | - | 1.27 | 0.40 | 1.45 |
| Falling number, s | 354 | 279 | 206 | 270 | 152 |
| Gluten content, % | 30.8 | 30.1 | - | - | - |
| Acidity, degrees N | 3.0 | 3.2 | 3.6 | 3.4 | |
| Colour | white with a yellowish tinge | | greyish-white interspersed with particles of shells | | |
| Odour | characteristic of flour, without extraneous | | | | |
| Taste | characteristic of flour, without extraneous | | | | |
| Count of microbes, CFU $g^{-1}$ | | | | | |
| - bacteria | 1,700 | 2,500 | 124,000 | 170,400 | 210,500 |
| - spore-forming bacteria | 20 | 800 | 2,000 | 200 | 500 |
| - yeast | 20 | 30 | 50 | 50 | 80 |
| - moulds | 130 | 1,000 | 2,200 | 300 | 400 |

However, it was established that, after baking in the wheat and rye wheat bread which had been prepared with sourdough both in the laboratory and in the bakeries themselves, there was no microbial contamination. The mould, bacteria, and yeast has not grown in the Petri dishes (Table 5). The studies confirm that the bread, after leaving the oven, is sterile in terms of the contamination of filamentous fungi, and any mould is a secondary infection (Lund et al., 1996; Afanasjeva, 2003; Cauvain, 2012).

**Table 5.** Microflora of bread immediately after the exit from the oven

| Number of micro-organisms, CFU $g^{-1}$ | Rye-wheat bread with sourdough | | Wheat bread with sourdough | Wheat bread without sourdough |
|---|---|---|---|---|
| | Dense rye sourdough (humidity 50%) | Yeast-free liquid rye sourdough (humidity 68%) | Dense (humidity 42%) | |
| Bacteria | not found | | | |
| Spore-forming bacteria | not found | | | |
| Yeast | not found | | | |
| Moulds | not found | | | |

To be able to determine the effect of sourdough on the quality of rye wheat bread and its stability in relation to microbial spoilage, an analysis was conducted of the biotechnological indicators of dense rye sourdough and liquid yeast-free sourdough. It was found (in Table 6) that different conditions of fermentation (involving the temperature and humidity of the sourdough, and the composition of micro-organisms) were affected in terms of their biotechnological parameters.

**Table 6.** Biotechnological indicators of different type's rye sourdough

| Biotechnological indicators | Sourdough | |
|---|---|---|
| | Dense rye sourdough | Yeast-free liquid rye sourdough |
| Acidity, degrees N | 14.9 | 20.0 |
| Lifting capacity, min. | 23 | - |
| Increase in volume, % | 75.0 | 45.5 |
| Quantity of alcohol, % | 1.8 | 0.8 |
| Volatile acids, % | 27.9 | 16.3 |
| Lactic acid, g $kg^{-1}$ | 6.0 | 11.0 |
| Acetic acid, g $kg^{-1}$ | 1.4 | 0.5 |

Dense rye sourdough had a good ability to lift (over the course of 23 mins), increasing in volume by 75%, and quite an amount of alcohol was also registered (1.8%) when compared to liquid yeast-free sourdough. This is due to the development of yeast in the dense rye leaven, which was used in the starter (part of the initial sourdough preparation), in contrast to liquid yeast-free sourdough with only lactic acid bacteria being used in the starter. The increase in volume (45.5%) in liquid yeast-free fermenting in relation to the activity shown by the heterofermentative lactic acid bacteria, *L brevis 1* and *L fermentum 34*, in producing $CO_2$ and the development of yeast cells from the flour. Sourdough also had different levels of acidity. There was a total of 1.8 times more

lactic acid and 2.8 times less acetic acid in yeast-free liquid rye sourdough when compared to the dense rye sourdough. Obviously, the accumulation of acetic acid contributes not only to heterofermentative lactic acid bacteria, but also to yeast.

When sterile slices of the bread known as 'Darnytskyi' with its dense rye sourdough were infected with the mould, *Penicillium chrysogenum*, it was found (in Table 7) that evidence of mould deterioration was not observed over the course of a total of 168 hours. After 168 hours of storage, the slices of bread in the Petri dishes became dried and hardened. At this stage monitoring was stopped. That bread which had been packaged into bags in a sterile environment was not mouldy after 336 hours of observation. That bread which had been packed in non-sterile conditions in the laboratory has exhibited signs of mould disease after a period of 192 hours.

**Table 7.** Effect of type of sourdough and its dosage when mixing the dough resistance to moulding of bread 'Darnitsky'

| Biotechnological indicators | Sourdough | | | | |
|---|---|---|---|---|---|
| | Dense rye sourdough | Yeast-free liquid rye sourdough | | | |
| Dough | | | | | |
| Part of the rye flour that was replaced by flour in the composition of sourdough, % | 25 | 15 | 20 | 25 | 30 |
| Bread | | | | | |
| Acidity, degrees N | 7.2 | 5.3 | 5.9 | 6.9 | 8.0 |
| Mass proportion of moisture, % | 47.0 | 46.8 | 46.8 | 46.8 | 46.8 |
| Quantity of alcohol, % | 0.8 | 0.6 | 0.6 | 0.4 | 0.4 |
| Volatile acids, % | 18.3 | 15.5 | 16.7 | 14.2 | 12.5 |
| Lactic acid, g $kg^{-1}$ | 4.8 | 4.7 | 4.8 | 5.2 | 5.4 |
| Acetic acid, g $kg^{-1}$ | 1.0 | 0.1 | 0.1 | 0.2 | 0.3 |
| Storage time before mould growth, hours: | | | | | |
| - sterile slices infected by *Penicillium chrysogenum* | no growth within 168h | 60 | 72 | 80 | no growth within 168h |
| Bread packed in a sterile environment | no growth within 14 days | | | | |
| Bread packed in a non- sterile environment | 192 | 168 | 192 | 192 | 192 |

On those slices of bread with a 15% liquid yeast-free rye sourdough instead of the rye flour in the recipe, mould was discovered after a period of sixty hours (see Table 6). When the amount of yeast-free flour in the composition of the sourdough is increased in favour of rye flour, to a total of 20% and 25% of the total flour content, the period of the growth for the mould increased to 72 and 80 hours respectively. The mass proportion of moisture did not vary enough from one kind of bread to another to be able to affect the relatively mould-free shelf life appreciably. Bread differs in acidity levels and in the content of acetic and lactic acids. It should be noted that the replacement in the recipe to the same levels of quantity (25%) with a dense rye sourdough and a liquid yeast-free rye sourdough bread had a close acidity reading of 7.2 and 6.9 deg, but the content of acetic acid in bread which was produced with liquid sourdough was five times less than it was

in bread produced with dense sourdough. When 30% of the quantity of rye flour in the recipe was replaced by yeast-free liquid rye sourdough, the development of mould in the bread was not observed within a period of 168 hours (which was also the case in bread which was produced with dense rye sourdough. But the acidity of the bread known as 'Darnytskyi' was at 8.0 deg – the maximum permissible according to normative documentation (State Standard of the Russian Federation, 1986). The bread had a pronounced acidic taste, one which would not be liked by consumers. Therefore it was found that the content levels of acetic acid which accumulated during the fermentation process effected the rate of the development of fungi on the bread, and that the lactic acid does not produce an effect. But evidently that other unidentified mould inhibitor can present itself in the fermented bread.

The effect of this method of bread-making upon the resistance of wheat bread to moulds was established (see Table 8). It was found that in bread slices with a dense wheat sourdough which was contaminated by *Penicillium chrysogenum*, the growth of mould colonies was observed between 18–24 hours later than in samples which had been prepared without sourdough and between 10–12 hours later than in samples with liquid yeast-free sourdough.

**Table 8.** Influence of the method of bread making on mould of wheat bread

| Biotechnological indicators | Without sourdough | Liquid yeast-free sourdough | Dense wheat sourdough |
|---|---|---|---|
| Sourdough | | | |
| Acidity, degrees N | | 5.7 | 6.3 |
| Mass proportion of moisture, % | - | 62 | 42 |
| Bread | | | |
| Acidity, degrees N | 1.0 | 1.2 | 1.4 |
| Mass proportion of moisture, % | 44.1 | 44.2 | 44.1 |
| Quantity of | | | |
| lactic acid, g $kg^{-1}$ | 1 | 3.5 | 3.5 |
| acetic acid, g $kg^{-1}$ | 0.1 | 0.1 | 0.3 |
| The storage time before mould growth, hours/day: | | | |
| - sterile slices infected by *Penicillium chrysogenum* | 30-36 | 40-48 | 50-60 |
| - bread packed in a non- sterile environment | 5-6 | no growth within 6-7 day | no growth within 10 days |

Bread without sourdough packed in non-sterile conditions had mould disease within a period of between 120–144 hours. Bread made with liquid yeast-free wheat sourdough had mould disease present within between 144–168 hours. Bread made with dense wheat sourdough did not exhibit mould disease within a period of 240 hours. The content of acetic acid in bread with a dense sourdough was higher than it was in other samples. Therefore, it can be seen that the use of wheat sourdough slows down the mould spoilage of wheat bread. Our research confirms other studies of the effect of a sourdough addition in relation to the development of bacterial and fungal spoilage, including variations of the percentage of sourdough inclusion (Denkova et al., 2014). The

application of the developed starters in terms of the production of wheat bread guarantees a longer shelf life.

## CONCLUSIONS

Studies have shown that the stability of bakery products in regard to mould spoilage depends on the type and quality of the sourdough, the technology being used, and the sanitary condition present at the point of production. Spore-forming and other bacteria, yeasts, and moulds were identified in the flour; however, no microbial contamination was found immediately after baking in the wheat bread and rye wheat bread when using this flour.

It was found that different fermentation conditions (regarding temperature, the humidity of the sourdough, or the composition of micro-organisms) affect the biotechnological parameters of the sourdough.

It was established that the content of acetic acid, which accumulates in bakery products during the fermentation of the sourdough, has an impact upon the speed of mould spoilage in bread. The lactic acid content does not effect the rate of development of fungi on the bread. But it is evident that other, unidentified, mould inhibitors can be present in the fermented bread.

It was proven that the use of sourdough in the preparation of bakery products when using rye and wheat flour allows their microbiological stability during storage to be increased.

## REFERENCES


Afanasjeva, O. 2003. Microbiology of baking. *St. Petersburg* 220 pp. (in Russian).

Cauvain, S.P. 2012. Breadmaking: Improving Quality. Second edition. *Woodhead Publishing* 598 pp.

Clarke, C.I., Schober, T.J. & Arendt, E.K., 2002. Effect of single strain and traditional mixed strain starter cultures on rheological properties of wheat dough and bread quality. *Cereal Chemistry* **79**, 640–47.

De Blackburn, C. 2008. Food Spoilage Microorganisms, *Woodhead Publishing* 736 pp.

Denkova, R., Ilieva S., Denkova, Z., Georgieva L., Yordanova, M., Nikolova, D. & Evstatieva, Y., 2014. Production of wheat bread without preservatives using sourdough starters. *Biotechnology & Biotechnological Equipment* **28**(5), 889–898.

Denkova, R., Ilieva S., Denkova, Z., Georgieva, L. & Krastanov, A. 2014. Examination of the technological properties of newly isolated strains of the genus Lactobacillus and possibilities for their application in the composition of starters. *Biotechnology & Biotechnological Equipment* **28**(3), 487–494.

Kosovan, A.P. 2008. The collection of modern technology of bakery products. In Kosovan, A.P. (ed.), *Moscow printing house*, 271pp (in Russian).

Legan, J.D. 1993. Mould spoilage of bread: the problem and some solutions. *International Biodeterioration & Biodegradation* **32**(1–3), 33–53.

Lund, F., Filtenborg, O., Westall, S. & Frisvad J.C. 1996. Associated mycoflora of rye bread. *Letters in Applied Microbiology* **23**(4), 213–217.

State Standard of the Russian Federation. 1996. Bread, rolls and buns. Methods for determination of acidity. GOST 5670-96, 5 pp (in Russian).

State Standard of the Russian Federation. 1986. Bread Darnitsky. Specifications. GOST 26983–86, 5 pp (in Russian).

*Agronomy Research* **15**(S2), 1358–1368, 2017

# Possibility of using reconstituted milk in manufacture of cheese with cheddaring and cheese curd stretching

N. Moiseev, E. Suchkova, N. Iakovchenko*

ITMO University, 191002, Lomonosova Street 9, Saint-Petersburg, Russia
*Correspondence: frack@mail.ru

**Abstract.** The use of reconstituted milk may significantly increase the possibility of cheese manufacture and limited irregular milk supplies for cheese making plants. Data collecting and analyzing revealed that there are cheese technologies with cheddaring and cheese curd stretching accompanied by partial replacement of natural milk by reconstituted. Therefore, the aim of this research is to develop the technology of cheese with cheddaring and curd stretching made from reconstituted milk as the main raw material. A comparative study of physicochemical characteristics of five dry milk powder samples obtained from different producers and natural milk has been carried out. The choice of reconstituted skimmed milk as the raw material is explained; its quality is assessed and the process parameters of milk reconstitution are chosen. It is recommended to combine holding of reconstituted skimmed milk and milk ripening. Cheddaring is known to be a fairly time-consuming process, the use of starter cultures during reconstituted milk ripening can intensify this process. The process of milk ripening has been carried out at 16 °C for 10 h using the manufacturer's recommended dosages of starter cultures. The best results have been obtained when Lyofast MOT 092EE is used. Milk ripening is found to be a very important operation for the production of cheese with cheddaring and cheese curd stretching made from reconstituted milk.

**Keywords:** cheese, pasta filata, reconstituted skimmed milk, stretchability, meltability

## INTRODUCTION

Pasta Filata type cheeses / Pizza type cheeses (PF cheese) are referred to cheeses with cheddaring and stretched curd and include such cheeses as Italian Mozzarella, Provolone, Kashkaval balkan, Mexican Oaxaca cheese, etc. These cheese varieties have the unique ability to stretch into thin strands when heated above 60 °C. Almost all pizza packagings and commercials focus on a cut pizza slice being lifted up to show the cheese melting and stretching out. Apparently, this cheese ability to stretch broadens consumer appeal for pizza and other food products containing PF cheeses (Gunasekaran & Ak, 2003).

The use of non-fat milk powder (NFMP) produced at low drying temperatures is of great interest for the production of PF cheeses with cheddaring and thermal processing. However, the production of PF cheeses from reconstituted skimmed milk (RSM) is a rather difficult task. During the manufacture of spray-dried milk, there are physical and chemical changes in milk components. These alterations are attributed, in particular, to whey proteins, as they have large amounts of hydrogen and easily cleavable covalent bonds. When unfolding protein globules during the denaturation process, the increase in

reactivity of sulfhydryl groups of cysteine and other sulfur-containing amino acids was observed. The aggregation of the denatured protein molecules occurs, involving hydrophobic interactions and the redox reactions of sulfhydryl groups forming disulfide bonds. Denaturation of β-lactoglobulin during heating is of practical interest. It forms stable complexes with κ-casein, preventing its losses in whey. Denatured β-lactoglobulin affects the properties of κ-casein, reducing the rennet coagulation properties, and the ability to stretch when heated (Wijayanti et al., 2014).

Avakimyan (2010) reported that a positive result was observed when NFMP in the amount of 50%by weight was used in the manufacture of cheeses with cheddaring and stretched curd. According to Gilles et al., (1982), unsatisfactory results were obtained when the low-heat NFMP was used as the raw material to produce Cheddar cheese. In this study RSM was standardized with fresh cream and dehydrated milk fat and then subjected to homogenization. The finished product had mealy texture and spongy body and had a metallic after–taste. In the manufacturing procedure for Cheddar cheese, the replacement of milk with NFMP up to 25% is a normal practice (Westergaard, 2010). NFMP was reconstituted with water to a total solids content range of 9–12%, held for 12 h and standardized with whole natural milk. According to Davide et al., (1993), it is possible to obtain mozzarella cheese with good meltability and stretchability using a combination of RSM (40–60%) and natural whole milk. Higher replacement ratio of RSM and natural milk has led to cheeses with higher moisture content and rigid curd.

In the literature studied there is information about the possibility of partial replacement of fresh milk with NFMP, however, no published information is available concerning the use of NFMP as the main raw material for PF cheese manufacture, therefore this research has been undertaken to fill in the gap.

The objective of this study was to develop the technology for producing cheese with cheddaring and curd stretching with the use of reconstituted NFMP as the raw material and to study the effect of RSM ripening on functional properties of cheese and the duration of cheddaring process.

## MATERIALS AND METHODS

The study included one control cheese group and three treatment cheese groups.

Control samples were made from unripened reconstituted skimmed milk. The treatment samples represented cheeses made from ripened reconstituted cheese milk.

### Preparation of cheese milk

PF cheeses can be produced only from low-heat NFMP (WPNI > 6 mg $g^{-1}$), as in this case, the less severe heat treatment is applied to milk while processing it to milk powder (Patel et al., 2007; Westergaard, 2010; Bylund, 2015). This study included 5 treatment samples of NFMP: 'Vamin', Russia; 'EuroSerum S.A.S. ', France; 'Lácteos La Cristina S.A. ', Argentina; 'Slutsk cheesemaking plant', Belarus; 'DMK Deutsches Milchkontor GmbH', Germany. On the basis of the results obtained, it was evident that only one sample of NFMP was matched to WPNI for low-heat milk powder, namely, 'DMK Deutsches Milchkontor GmbH' (36.6 ± 1.2% total protein, 4.0 ± 0.5% total moisture, 1.0 ± 0.3% total fat, 6.3 ± 0.2 mg N $g^{-1}$ WPNI, 0.5 ± 0.1 ml, solubility index).

The NFMP reconstitution was carried out using softened water (0.058°dH) at a temperature of 45 °C. The NFMP was dissolved using shear pump RPA–5 looped by

connecting the reservoir avoiding dry running and stirred for 10 min until completely dissolved. The total solids content of RSM was 10% (w/v). RSM was subjected to vat pasteurization at 63 °C for 30 min. These heat treatment parameters are recommended for milk and milk products (Clark et al., 2008).

To produce treatment samples of cheeses the following lyophilized direct vat set cultures recommended for PF cheese production were used: CHOOZIT ™ ALP LYO, France, 100DCU; AiBi LcLs30.11, Russia; Lyofast MOT 092EE, Italy, 10UC.They contain mesophilic and thermophilic microorganisms. Mesophilic starter cultures are necessary for milk ripening. The procedure of pack opening was as follows: the pack edge and scissors were processed using a swab dipped in 70% alcohol solution. The amount of starter cultures was ¼ of the pack following the recommendations provided by the manufacture (1 pack per 1000–2000 l of milk). The optimum temperature of the milk ripening is in the range of 10–20 °C. It is the lowest temperature limit for growth of mesophilic lactic acid microorganisms (Robinson, 2002). This research suggested RSM ripening at a temperature of 16 °C for 10 h. The technological parameters were indicated by Avakimyan (2010).

Cheddaring process is a rather long-term operation and can take up to 5 hours depending on the types of microorganisms, used as a starter, and cheddaring temperature. Therefore, RSM ripening can intensify this process, i.e. reduce the time needed to reach the required values of pH.

Cheese milk for the production of unripened milk cheese samples was normalized after milk reconstitution and pasteurization. For the treatment cheese samples cheese milk was standardized after the RSM milk ripening.

The fat content of RSM was standardized to 2.8% with pasteurized cream (15% fat). The cream was subjected to heat treatment in 'Prinevskoe' Student farm, Russia, Leningrad region, Vsevolozhsk district. Normalization was carried out using RPA–5 pump looped by connecting the reservoir at a temperature of 45 °C. Standardized mixture formulation is presented in Table 1.

**Table 1.** Formulation for the standardized mixture

| Ingredients | Amount, kg | Total solids, % | Fat, % |
|---|---|---|---|
| NFMP | 25.0 | 96.0 | 1.0 |
| Water | 180.0 | - | - |
| Cream | 45.0 | 22.0 | 15.0 |
| Total | 250.0 | 13.5 | 2.8 |

**Cheese making process**

The cheese making process (Fig. 1) was performed using medium-scale manufacturing conditions at the 'OOO Sfera', Saint – Petersburg, Russia. The manufacturing process was carried out using cheese equipment 'DR. GUBER', Russia. It produced twelve cheese batches (1 batch per day).

**Cheese made from ripened milk**

After ripening and standardization, the prepared mixture (250 l) was cooled to 38 °C. Calcium chloride in the amount of 50 g per 100 kg of the mixture as a 40% solution prepared with distilled water was added.

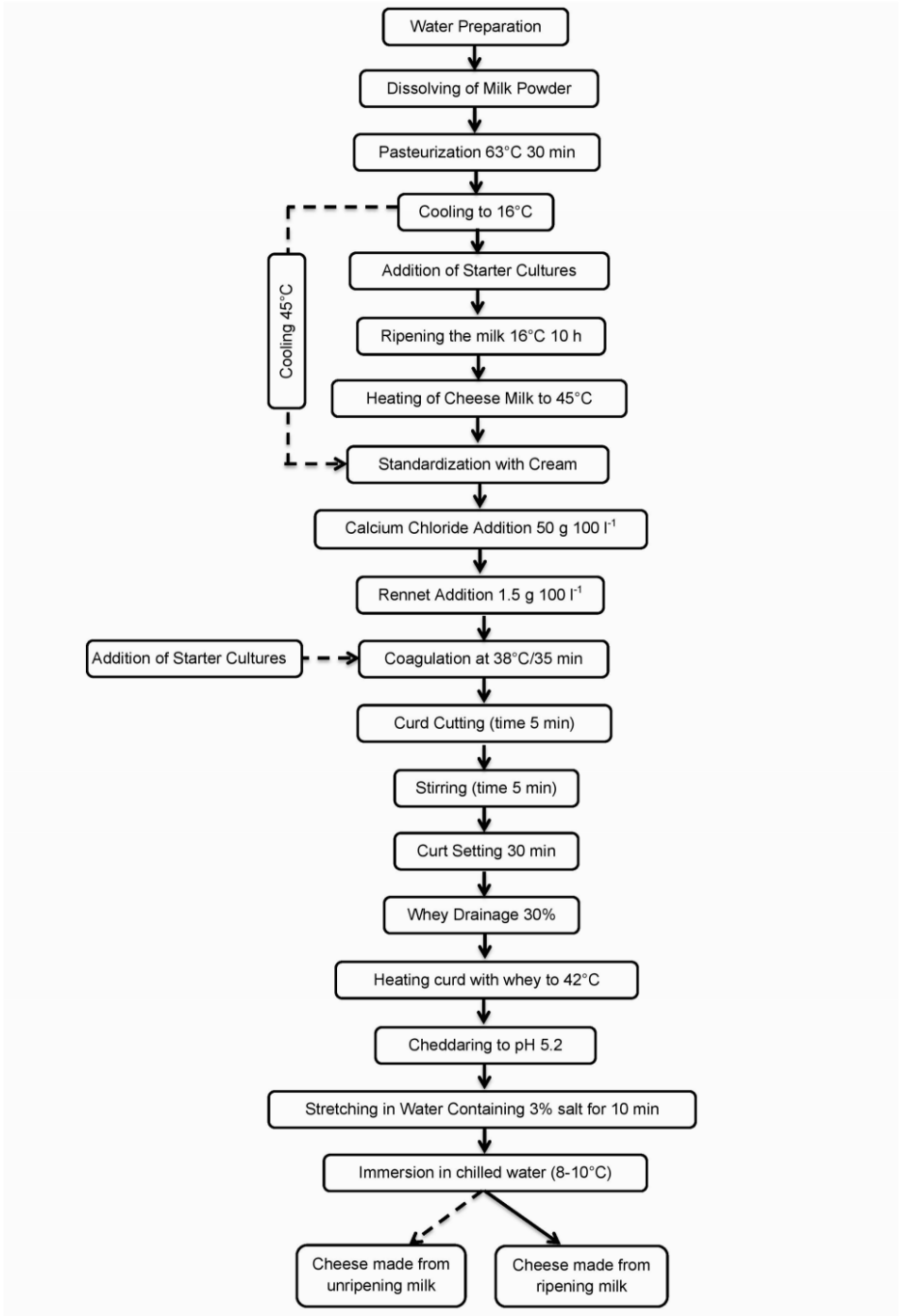



**Figure 1.** Flow chart for manufacture of pasta filata cheese.

Rennet ('Renmax 2100 Granular', MAYASAN Food Industries A.S., Turkey, 1.5g 100 $l^{-1}$) was added according to the following technique: 4.5 g of rennet was dissolved in 250 $cm^3$ of warm distilled water an hour before use. The mixture was coagulated at

38 °C for 35min. The end of coagulation was defined as the point at which the curd was firm enough to be cut during cheese making. It was determined by inserting the spatula into coagulum at 45°, gently lifting the spatula and observing the curd split. A sharp, clean split indicates that the curd is ready for cutting. The resulting curd was cut into 10 $mm^3$ cubes with vertical and horizontal curd knives and cheese grain formation was carried out to promote whey expulsion. Then, whey was drained partially (30%) and the curd particles were subjected to cheddaring at 42 °C until the pH of the curd reached 5.1–5.2 with subsequent hand formation of balls with the diameters of 5–8 cm in brine solution (3%) at 65 °C. After these operations, the obtained cheeses were cooled by soaking in water for 5 minutes with a temperature ranged from 8 °C to 10 °C.

**Cheese made from unripened cheese milk**

The samples were produced according to the same process of ripened milk cheese sample manufacture, except that the starter culture was added after the standardization process and cooling to 38 °C.

The samples used in this study for comparing stretchability and meltability were as follows: control sample – Mozzarella (Galbani, Russia), purchased by the researchers at a local food market with a full–service cheese department which allowed for proper cheese storage; sample 1 – cheese made from standardized ripened reconstituted milk; sample 2 – cheese made from standardized unripened reconstituted milk.

**Chemical analyses and functional tests**

Titratable acidity (TA) was determined according to AOAC method 947.05 (1995). TA was determined by titration of a known amount of milk sample with 0.1 N NaOH using phenolphthalein as indicator. TA was expressed as a percentage of lactic acid. Total moisture content of NFMP was determined according to ISO 5537:2004. pH values were measured using pH-meter (pH-410, Akvilon Company, Russia). WPNI was determined by GEA method (GEA Niro Method No. A 21 a) by Kjeldahl method using the automated analyzer Kjeltec System 1030 (FOSS Analytical AB, Sweden) and spectrophotometer UV-1800 (Shimadzu, Japan). Solubility index was determined according to ISO 8156:2005. Total fat in NFMP was determined according to ISO 1736:2008. The amount of total nitrogen was determined as the amount of soluble nitrogen divided by the total nitrogen amount and expressed as a percentage. Total nitrogen and soluble nitrogen contents of the cheese samples were measured according to ISO 27871:2011. Total protein was expressed as total nitrogen content multiplied by 6.38 (Moatsou et al., 2002). Moisture content of cheese samples was determined according to ISO 5534:2012. Total protein was determined by Kjeldahl method according to ISO 8968-1:2014. Fat in dry matter was determined by gravimetric method according to ISO 1735:2004.

Laboratory balance DL-120, (A&D, Japan) was used for accurate measurements of weight.

The meltability was determined according to the method described by Richouxet et al., (2001), which was derived from the Schreiber test, and was expressed as the percent increase in the cheese disc diameter after heating at 225 °C for 3 min.

Stretchability test was carried out according to USDA (1980) and (Caro et al., 2011), the technique used was as follows: cheese samples (30 g) were placed in the center of the corn tortillas and heated in a microwave oven at 1,650 W for 30 sec before the

evaluation. A stainless steel fork with 4 tines was lowered into the melted cheese to a depth of 3 mm at 45-degree angle, then the fork was pulled slowly and vertically for 5 sec. The distance, which cheese strands were lifted to, until they broke, was defined using metric measuring tape. The results were measured using 9-point structured scale (from 1 = 10 cm to 9 = 90 cm, respectively). To minimize inaccuracy, a panel consisted of 5 members was formed. Each member conducted 5 tests and calculated the mean value of a data set. As a result, the mean value of 5 observations of all members was taken.

**Statistical analyzes**

All experiments were performed at least in triplicate (unless stated otherwise) and the results were expressed as the mean values ± standard deviation. Statistical processing of data was carried out using computer programs Microsoft Office Excel 2010. The Bonferroni t-test was used to determine significant differences among means, and differences were described as significant only at $p < 0.05$.

## RESULTS AND DISCUSSION

According to the data presented in Table 2, TA mean values for RNFMP before and after ripening showed that the application of AiBi LcLs30.11 and Lyofast MOT 092EE starter cultures increased TA mean values by 0.036 ± 0.03 %LA, while the use of CHOOZIT™ ALP TA led to an increase in TA mean values by 0.041 ± 0.06 %LA. The average pH values showed an increase with the use of AiBi LcLs30.11 by 0.23 ± 0.03 and by 0.25 ± 0.03 and 0.27 ± 0.06 for Lyofast MOT 092EE and CHOOZIT™ ALP samples, respectively. Obvious differences in the starter cultures at this stage were not found.

**Table 2.** Changes in TA of RNFMP samples (± standard deviations)

| Starter Culture | Before ripening | | After ripening | |
|---|---|---|---|---|
| | Titratable acidity, %LA | pH | Titratable acidity, %LA | pH |
| CHOOZIT™ ALP | 0.153 ± 0.03 | 6.71 ± 0.10 | 0.194 ± 0.06 | 6.44 ± 0.12 |
| AiBi LcLs30.11 | 0.153 ± 0.03 | 6.71 ± 0.10 | 0.189 ± 0.03 | 6.48 ± 0.10 |
| Lyofast MOT 092EE | 0.153 ± 0.03 | 6.71 ± 0.10 | 0.189 ± 0.03 | 6.46 ± 0.11 |

When AiBi LcLs30.11 was used, active acidity (pH) decreased very slowly and after 160 minutes it reduced to a value of 5.87 as compared to other samples that reached 5.13 and 5.17 for Lyofast MOT 092EE and CHOOZIT ™ ALP LYO, respectively (Fig. 2). Apparently, these results were obtained due to the low quality of the starter culture used or the attack of bacteriophage and poor resistance of the starter culture strains to the action of bacteriophage. No significant differences were observed in the activity of Lyofast MOT 092EE and CHOOZIT ™ ALP LYO ($p < 0.05$).

The quality of the cheese is directly affected by proteolysis. This is due to the disruption of the casein matrix under the action of proteolytic systems produced by bacterial cells. As a result, this process causes the improvement in cheese melting, but it becomes less stretching and is easily disrupted. Therefore, the stronger the proteolytic cleavage in the cheese curd, the lower its stretchability (McSweeney, 2007; Avakimyan,

2010). In this regard, the degree of cheese curd proteolysis was studied by investigating the process of proteolysis product accumulation in cheese curd at the end of cheddaring stage using Lyofast MOT 092EE and CHOOZIT ™ ALP LYO.

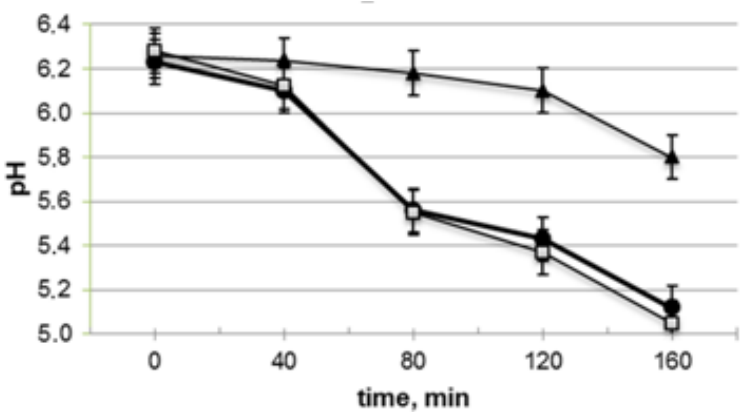



**Figure 2.** Changes in pH values (± standard deviations) during cheddaring (● – CHOOZIT™ ALP; ▲ –LcLs30.11; □ – Lyofast MOT 092EE).

In terms of the result it was found that the analyzed samples differed in their average total soluble nitrogen (Fig. 3) content which reached 17.5 ± 0.8% and 13.1 ± 0.6% in cheese curd with the use of CHOOZIT ™ ALP LYO and Lyofast MOT 092EE, respectively. The results revealed that the most proteolysis product accumulation was in the cheese curd containing CHOOZIT ™ ALPLYO, which has a higher proteolytic activity compared to Lyofast MOT 092EE. This activity manifested itself in the cleavage of casein with the formation of free amino acids, peptides and nitrogen.

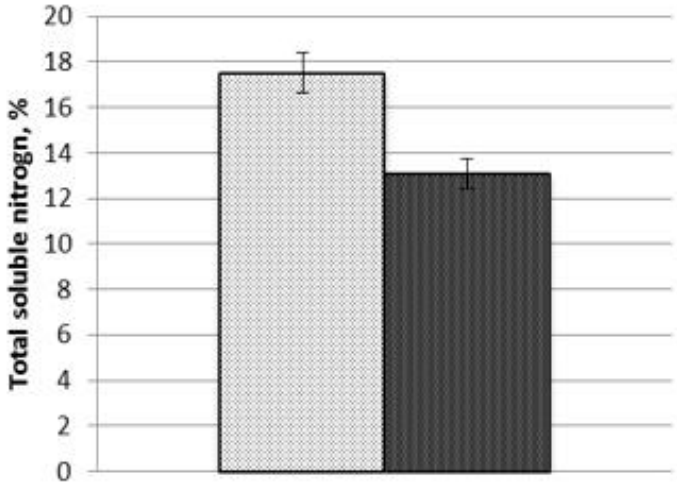



**Figure 3.** Total soluble nitrogen content, % (± standard deviations) in cheese curd at the end of cheddaring (▒ -CHOOZIT ™ ALP LYO, ▓ - LyofastMOT 092EE).

Thus, the findings of this research allowed to draw a conclusion that the use of Lyofast MOT 092EE for obtaining elastic and more plastic cheese, as well as for cheddaring process intensification, was more efficient.

The next step of the study was to determine the influence of milk ripening on the cheddaring process duration. From Fig. 4 it can be seen that the duration of cheddaring process decreased by about 50 minutes until pH reached a pH of 5.2. This decrease could be due to the development of mesophilic lactic acid bacteria during ripening and preparing favorable conditions for lactic acid microorganism growth (Law et al., 2010).

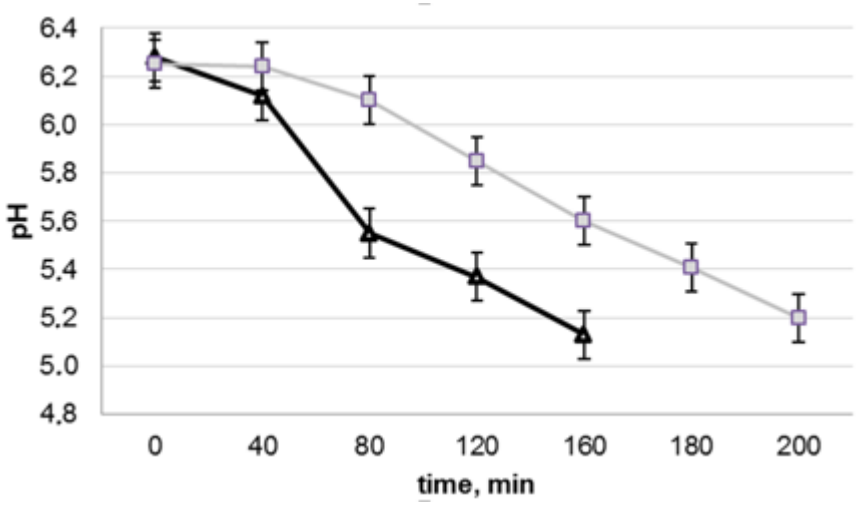



**Figure 4.** Changes in pH values (± standard deviations) during cheddaring process (▲ – cheese made from ripened milk; □ – cheese made from unripened milk).

It can be seen from Table 3 that physico-chemical parameters of the samples did not differ significantly ($p < 0.05$).

**Table 3.** Physicochemical parameters of cheese samples (± standard deviations)

| Cheese composition | Control | Sample 1 | Sample 2 |
|---|---|---|---|
| Moisture (g 100 $g^{-1}$) | 56.4 ± 2.2 | 52.2 ± 4.5 | 55.5 ± 2.6 |
| Fat (g 100$g^{-1}$) | 19.6 ± 1.0 | 22.1 ± 1.2 | 21.5 ± 1.0 |
| Protein (g 100 $g^{-1}$) | 21.5 ± 0.9 | 22.2 ± 1.5 | 20.5 ± 1.2 |
| pH | 5.15 ± 0.10 | 5.2 ± 0.12 | 5.15 ± 0.12 |

As shown in Figs 5 and 6, the control sample represented the best results for stretchability and meltability (6.5 ± 0.4 points and 48.3 ± 4.1%, respectively). Sample 1 also demonstrated good melting and stretching properties (35.0 ± 3.2% and 5.1 ± 0.2 points) in contrast to sample 2, which showed poor ability to stretch (0.5 ± 0.1 points).

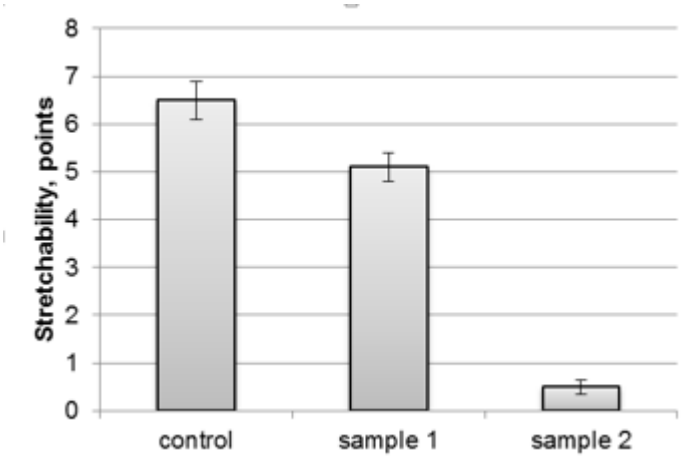



**Figure 5.** Stretchability mean values (± standard deviations) of different cheese samples.

The inferior melting and stretching can be caused by both changes in the protein molecule of casein and salt balance occurring during the drying process. Apparently, holding of RSM with the use of starter cultures for at least 10 hours contributes to casein unfolding and maximum recovery of its native properties.

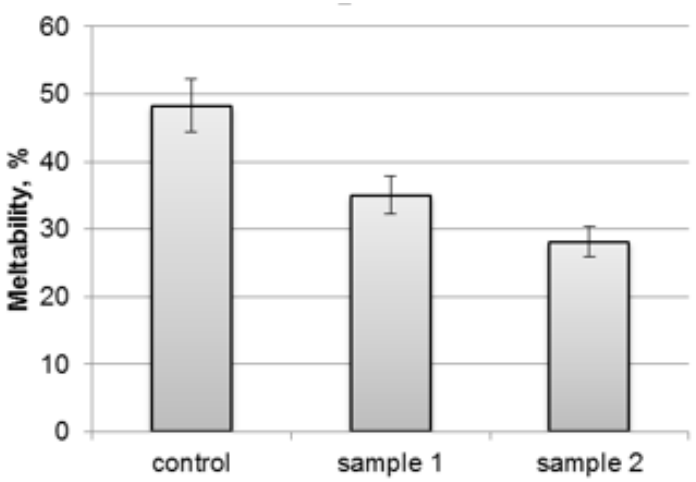



**Figure 6.** Meltability mean values (± standard deviations) of cheese samples.

In addition, the following physicochemical and colloidal properties are changed: the oxidation-reduction potential is reduced, the content of polypeptides is increased, and some of the calcium salts become soluble. These changes contribute to decreased stability and dispersity of casein micelles and increase the efficiency of the demineralization of calcium salts. When unripened reconstituted milk is used, the cheese ability to stretch into thin and elastic strands, when melted, decreases significantly ($p < 0.05$).

Sample 2 cannot be legally called PF cheese, as it does not correspond to the requirements for pizza cheese provided by the USDA (1980) which has stated that the cheeses of this type must be stretched not less than 30 cm, when heated.

## CONCLUSIONS

It is necessary to carry out special preparation of RNFMP for the manufacture of PF cheese. This special operation includes the reconstitution of NFMP and ripening of RSM with the use of starter cultures containing different microorganism strains.

For PF cheese production made from NFMP it is necessary to hold and ripe milk for at least 10 hours.

The possibility of reducing the duration of cheddaring process by RSM ripening for about 50 min is shown.

The necessity of RSM ripening and its influence on stretchability and meltability of the finished product are demonstrated. The ripening of RSM increases stretchability and meltability by 90.2% and 19.7%, respectively, compared to the cheese produced from unripened RSM.

More research is needed to study diligently the influence of RSM holding and ripening on functional properties and quality of PF cheeses.

ACKNOWLEDGEMENTS. This work was financially supported by the Government of the Russian Federation, Grant 074–U01.

## REFERENCES

AOAC. 1995. *Official methods of analysis*. 16th Ed. Arlington: AOAC International.

Avakimyan, A.B. 2010. *Technology development and investigation of smoked cheeses with cheddaring and thermomechanical processing of cheese mass.* Voronezh State Academy of Technology, Voronezh, 245 pp. (in Russian).

Gilles, J., Lawrense, R.C., Czulak, J., Conochie, J. & Hammond, L.A. 1982. Cheddar cheese based on reconstituted skim milkpowder. *JDF Bulletin* **6**, 33–35.

Bylund, G. 2015. *Dairy processing handbook*. Tetra Pak Processing Systems AB, Lund, 482pp.

Caro, I., Soto, S., Franco, M.J., Meza-Nieto, M., Alfaro-Rodríguez, R.H. & Mateo, J. 2011. Composition, yield, and functionality of reduced-fat Oaxaca cheese: Effects of using skim milk or a dry milk protein concentrate. *J. Dairy Sci*. **94**(2), 580–588.

Clark, S., Costello, M., Drake, M. & Bodyfelt, F. 2008. *The Sensory Evaluation of Dairy Products*. Springer, New York, 576 pp.

Davide, C.L., Sarmago, I.G. & Rocafort, R.F. 1993. Developments of natural part skim mozzarella-type cheese for the pizza industry. *Philippine Agric*. **76**, 21–34.

GEA Niro. 2009. A/S NiroAtomizer: Whey Protein Nitrogen Index. GEA Niro Method No. A21a. Accessed Jan. 3, 2016. http://www.gea.com/en/binaries/A%2021%20a%20-%20Whey%20Protein%20Nitrogen%20Index_tcm11-30931.pdf

Gunasekaran, S. & Ak, M.M. 2003. *Cheese Rheology and Texture*. CRC Press, Washington, 437 pp.

ISO 1735:2004.Cheese and processed cheese products – Determination of fat content – Gravimetric method.

ISO 1736:2008. Dried milk and dried milk products – Determination of fat content – Gravimetric method.

ISO 27871:2011. Cheese and processed cheese – Determination of the nitrogenous fractions

ISO 5534:2012. Cheese and processed cheese – Determination of the total solids content.
ISO 5537:2004. Dried milk – Determination of moisture content (Reference method)
ISO 8156:2005. Dried milk and dried milk products – Determination of insolubility index.
ISO 8968-1:2014. Milk and milk products – Determination of nitrogen content – Part 1: Kjeldahl principle and crude protein calculation.
Law, B.A. & Tamime, A.Y. 2010. *Technology of Cheesemaking*. Wiley-Blackwell, 512 pp.
Moatsou, G., Massouras, T., Kandarakis, I. & Anifantkis, E. 2002. Evolution of proteolysis during the ripening of traditional feta cheese. *Lait* **82**, 601–611.
McSweeney, P.L.H. 2007. Cheese Problems Solved. *Woodhead Publishing, Abington*, 424 pp.
Patel, H.A., Anema, S.G. Holroyd, S.E., Singh, H. & Creamer, L.K. 2007. Methods to determine denaturation and aggregation of proteins in low-, medium and high–heat skim milk powders. *Lait* **87**, 251–268
Robinson, R.K. 2002. *Dairy Microbiology Handbook. The Microbiology of Milk and Milk Products*. John Wiley&Sons, New York, 765 pp.
Richoux, R., Roset, G., Famelart, M.H. & Kerjean, J.R. 2001. Diversity of some hot functional properties of French Emmental (Diversité de quelques pro-priétésfonctionnelles à chaud de l'Emmentalfrançais). *Lait* **81**, 547–559 (in French).
USDA. 1980. USDA *Specifications for Mozzarella Cheeses.* Agricultural Marketing Service, USDA, Washington, DC.
Westergaard, V. 2010. *Milk Powder Technology. Evaporation and Spray Drying.* GEA Niro, Copenhagen, 339 pp.
Wijayanti, H.B., Bansal, N. & Deeth, H.C. 2014. Stability of Whey Proteins during Thermal Processing: A Review. *Comprehensive Reviews in Food Science and Food Safety* **13**(6), 1235–1251.

*Agronomy Research* **15**(S2), 1369–1374, 2017

# Development of a rapid method for determination of gluten content in wheat flour

V. Nazarova[1,*] and O. Zhdanova[1]

[1]ITMO University, Faculty of Food Biotechnologies and Engineering, Lomonosov St. 9, 191002, Saint-Petersburg, Russia
*Correspondence: vvnazarova@yandex.ru

**Abstract**. Gluten content is one of the most important factors of the quality of wheat flour. Bread price depends on the quality and quantity of gluten. For the professionals of baking industry it is important to quickly and efficiently determine gluten content in wheat flour. Gluten content is commonly defined by manual or automated washout. The present study aimed to develop a rapid method for determination of gluten content in wheat flour by electrophysical method and compare it with the other standard methods (ISO 21415–1:2006, ISO 21415–2:2015). The method is based on determination of permittivity of flour, which varies depending on the amount of free water produced by heating and correlated with the content of gluten. The methodology of research included the use of wheat flour samples (gluten content in the range of 23.0% and 32.0% and flour humidity to 15.0%). The heating temperature of the analysed flour samples was in the range of 30 ºC and 70 ºC. Mathematical processing of the results of the experiment allows to establish the relationship between gluten content and capacitance of flour. The proposed method makes it possible to reduce the time of analysis by reducing the number of operations within the analysis and the influence of subjective factors comparing to manual and automatic washing method a gluten flour (ISO 21415–1:2006, ISO 21415–2:2015).

**Keywords:** gluten content, rapid determination, permittivity.

## INTRODUCTION

Gluten is an indicator of quality not only of flour but also of a grain. Flour can be used in various fields of food industry depending on the gluten content of wheat (Kazakov, 2001). Control of gluten content and its quality is carried out in the production of wheat flour and bakery products. It is important to collect this information as soon as possible (Zannini et al., 2012).

Therefore, many countries are developing rapid methods for determining content of gluten based on the various approaches (Puchkov et al., 2005). Wet gluten content is determined by washing dough with water or other solution under certain conditions in order to remove starch and other soluble components of the sample (Mis, 2000). Elastic mass that remains after washing is wet gluten. Operating condition and type of solution bears heavy influence on the results. It is recommended to use a solution of NaCl (2%) to neutralize the effect of different minerals normally found in drinking water. Also, washing time should not exceed 30 minutes.

There is a device called Glutomatic 2200 included in the International Standards

AACC–38–12, ICC–155, ICC–158, but the device is expensive and is not always possible to implement. The standard method for determining the quantity and quality of gluten (ISO 21415–1:2006**,** ISO 21415–2:2015) is time consuming and requires a significant amount of drinking water.

The aims of following research were the development of a rapid method for determination of gluten content in wheat flour using capacitive method and comparison of the proposed method and standard method **(**ISO 21415–1: 2006, ISO 21415–2: 2015).

## MATERIALS AND METHODS

This study used 5 samples of high grade bakery wheat flour with different gluten content. Wet gluten content was found according to the method ISO 21415–1:2006 (Table 1). Moisture content was determined according to the method ISO 712:2009. All tests were carried out three times and the mean values were noted with the standard deviation.

To obtain the results using the following method it is required to: prepare the flour samples with different gluten content and different humidity; provide a constant density of the sample; and provide a temperature control system for measuring gluten content in heated flour sample.

**Table 1.** Gluten content of wheat flour (ISO 21415–1:2006)

| Flour sample | Gluten content, % |
|---|---|
| 1 | 23.0 |
| 2 | 27.6 |
| 3 | 29.3 |
| 4 | 30.5 |
| 5 | 32.0 |

The flour samples with a specific gluten content were put in the capacitive transducer. Flour was heated to 70 ± 5 °C. The values of capacitance were measured every 10 seconds in the range of 30–70 °C. Considering a possible gluten protein denaturation heating temperature was considered as 70 ± 5 °C (Sherwy & Tatham, 1997; Moick & Rogova, 1988). The values of electrical capacitance of flour were determined on the experimental setup shown in Fig. 1.

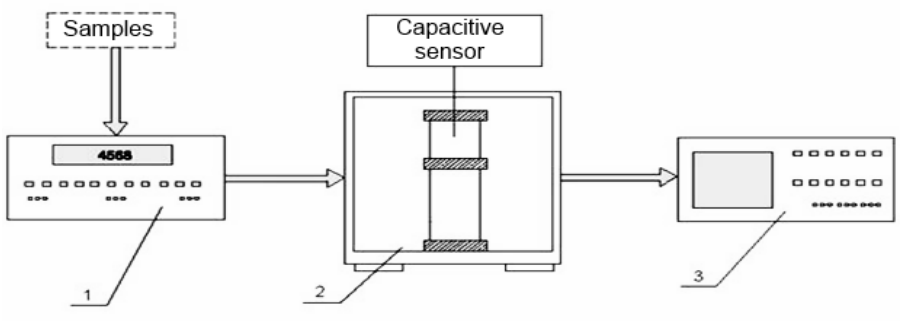



**Figure 1.** The experimental setup. 1 – moisture meter ELVIZ–2, 2 – oven SNOL58/350 with a capacitive sensor, 3 – capacitance meter ET–20.

## RESULTS AND DISCUSSION

The diagram was plotted to determine gluten content in flour using capacitive method. It shows relationship between capacitance and temperature of 5 flour samples. Flour capacitance is greater the more the gluten content in wheat flour. Thus, electric capacitance depends on the gluten content of flour. As gluten and starch hold bound moisture then translation of moisture bounded by gluten in the free state by heating flour samples causes changes in the flour capacitance (Fig. 2) (Duckuort, 1980; Kazakov & Karpilenko, 2005).

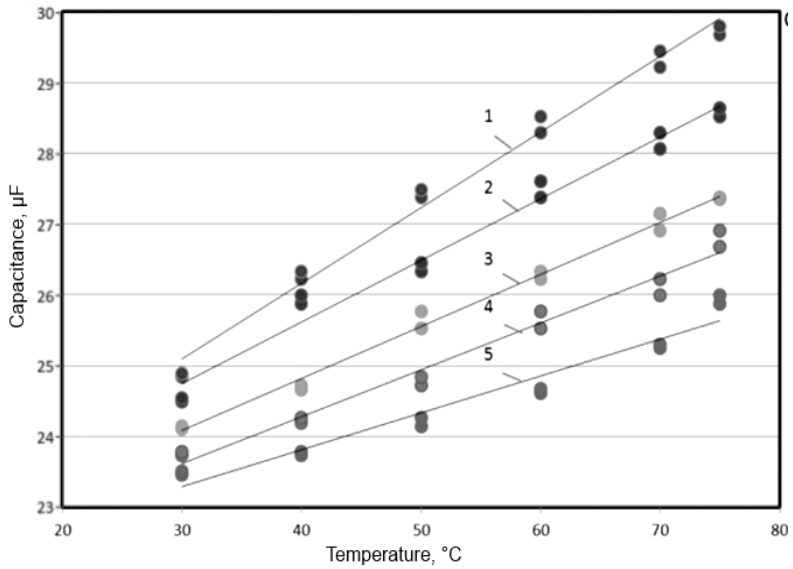



**Figure 2.** Relationship between capacitance and temperature. Gluten content of the samples: 1 – 32.0%, 2 – 30.5%, 3 – 29.3%, 4 – 27.6%, 5 – 23.0%.

To calculate gluten content in flour using capacitive method we have moved from electric capacitance to the permittivity, which value is not depending on sensor's size. Knowing the capacitance of an empty sensor and capacitance of a sensor filled with the flour sample we moved to the value of the dimensionless permittivity. Further, the relationship between permittivity and temperature was obtained, which also confirms that permittivity which grows with the temperature is greater the more gluten and starch content (Mikulovich & Dolgopolov, 1991). Gluten content can be calculated using the formula:

$$\varepsilon(70 flour\ calc.) = 1.68 + 0.018K_{gluten},$$

where: $\varepsilon$ – permittivity of wheat flour at a temperature 70 ± 5 °C; $0.018K_{gluten}$ – gluten content.

Using the experimental data we discovered the relationship between permittivity of wheat flour and gluten content, which enabled development of the regression equations.

This served as foundation for determining gluten content in wheat flour depending on permittivity of wheat flour, as shown in Fig. 3.

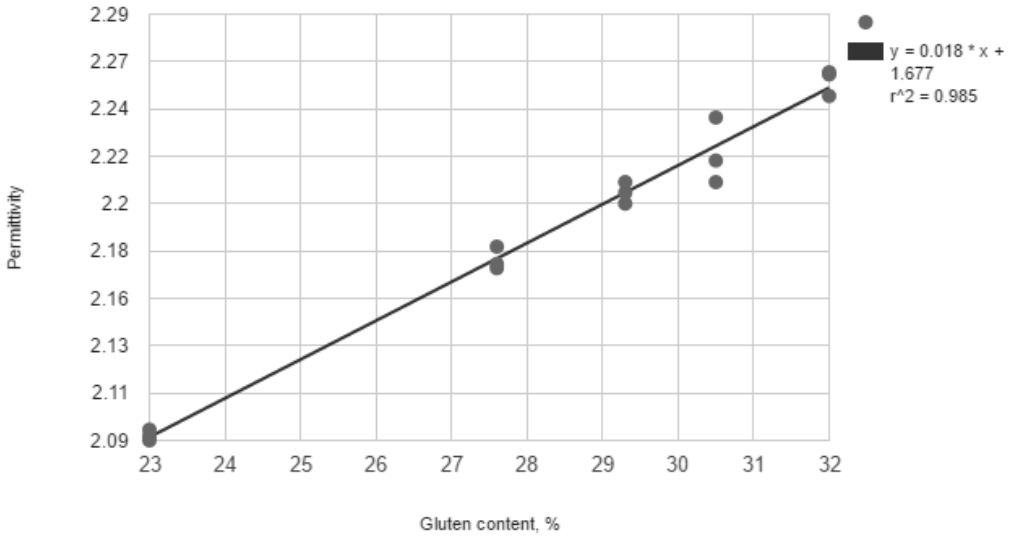



**Figure 3.** The relationship between permittivity and gluten content.

Table 2 shows comparison of gluten content values obtained using the proposed method and gluten content values obtained using the standard method ISO 21415–1:2006. The comparison shows that the proposed method gives comparable results to the standard method.

**Table 2.** Comparison of methods for determination of gluten content in wheat flour

| Flour sample | Gluten content, % (standard method) | Gluten content, % (proposed method) |
|---|---|---|
| 1 | 23.0 | 23.15 |
| 2 | 27.6 | 27.48 |
| 3 | 29.3 | 29.15 |
| 4 | 30.5 | 30.55 |
| 5 | 32.0 | 31.8 |

The closest analogue to the technical nature of the proposed method are ISO 21415–1:2006 ‘Wheat and wheat flour. Gluten content. Part 1: Determination of wet gluten by a manual method’ and ISO 21415–2:2015 ‘Gluten content. Part 2: Determination of wet gluten and gluten index by mechanical means’.

As shown in Table 3, the manual method comprising nine successively stipulated operations to determine the gluten content in the flour take 70 minutes and determination of gluten content by mechanical means take 20 minutes.

The proposed method takes no more than 10–12 minutes to conduct the experiment in three operations.

**Table 3.** The comparison of methods of determination of wet gluten content of wheat flour

| ISO 21415–1:2006 | | ISO 21415–2:2015 | | Proposed Method | |
|---|---|---|---|---|---|
| Operation | Time, min | Operation | Time, min | Operation | Time, min |
| Weighing flour | 1 | Weighing Flour | 1 | Placing flour samples in the sensor | 1 |
| Dosing water | 1 | Dosing water | 1 | Heating flour samples | 10 |
| Doughing | 5 | Doughing | 3 | Determination of gluten content | 1 |
| Binning of dough | 21 | Binning of dough | 10 | | |
| Washing out | 30 | Squeezing of dough and dividing it into 5–6 pieces | | | |
| Pressing between palms (removing excess moisture from the sample) | 3 | Main washing of gluten | 3 | | |
| Weighing | 2 | Weighing | 2 | | |
| Washing and pressing | 7 | | | | |
| Weigh | 2 | | | | |
| Total time | 70 | | 20 | | 12 |

## CONCLUSIONS

The results obtained in this work indicated the prospects of the developed method of determining the gluten content in wheat flour.

The developed method of determination of gluten content can be used in flour and bakery processing industry. Implementation of this method opens up prospects for a rapid control of determining the gluten content in the flour by capacitive method.

## REFERENCES


Duckuort, R.B. 1980. *Water in food.* Food Industry, Moscow, 376 pp. (in Russian).

ISO 21415–1:2006 'Wheat and wheat flour. Gluten content. Part 1: Determination of wet gluten by a manual method'.

ISO 712:2009 'Cereals and cereal products – Determination of moisture content – Reference method'.

ISO 21415–2:2015 'Gluten content. Part 2: Determination of wet gluten and gluten index by mechanical means'.

Kazakov, E.D. 2001. Composition, structure and properties of gluten. *Bakery* **9**, 18–19. (In Russian).

Kazakov, E.D. & Karpilenko G. 2005. *Biochemistry of grain and grain products.* GIORD, Saint–Petersburg, 232 pp. (in Russian).

Mikulovich, T.P. & Dolgopolov, V.P. 1991. *Vegetable protein.* Agropromizdat, Moscow, 325 pp. (in Russian).

Mis, A. 2000. Some methodological aspects of determining wet gluten quality by the glutomatic method (a laboratory note). *International Agrophysics* **14**, 263–267.

Moick, I.B. & Rogova I.A. 1988 *Thermo and hygrometry food.* Agropromizdat, Moscow, 303 pp. (in Russian).
Puchkov, L.I., Polandova, I.D. & Matveeva, I.V. 2005. *Bread technology*. GIORD, Saint–Petersburg, 559 pp. (in Russian).
Sherwy, P.R. & Tatham, A.S. 1997. Disulphide bonds in wheat gluten proteins. *Journal of Cereal Science* **25**(3), 207–227.
Zannini, E., Julie, M., Jones, S., Renzetti, K. & Arendt, E.K. 2012. Functional replacements for gluten. *Annual Reviews of Food Science and Technology* **3**, 227–245.

*Agronomy Research* **15**(S2), 1375–1385, 2017

# Effects of various raw ingredients on bread quality

N. Naumenko,* A. Paymulina, A. Ruskina and V. Khudyakov

South Ural State University, Higher School of Medicine and Biology, Department of Food and Biotechnology, 85 Lenina Avenue, 454080 Chelyabinsk, Russia;
*Correspondence: naumenko_natalya@mail.ru

**Abstract**. The purpose of the current research is to study the mechanisms behind how various raw ingredients affect the quality of bread. The objects of the research were the flour used in making the bread (consisting of gluten at 28.5%, and with an ash content of 0.55), with no added fats; tap water or activated water (treated in a USTA-0.4/22 OM ultrasonic processor (Volna, Russia), operating at a frequency of 22 ± 1.65 kHz and at 30% of maximum output power (400W) for mixing dough); and plant extract additives based on stevioside and fucoidan (fully replacing the sugar). Included in the analysis were the effects of using activated water and combined plant extract additives on organoleptic qualities (appearance, crust colour, crumb condition, taste, stickiness during mastication, and friability), as well as the physical and chemical qualities (moisture content, porosity, and acidity). Yeast activity was studied in dough which had been produced using activated water and combined plant extract additives. An Altami-136T optical microscope (Altami, Russia) was used to study the activity of yeast cells. The effects of activated water and combined plant extract additives were analysed by examining the microstructure. Microscopic studies were carried out using a Jeol JEM-2100 electron microscope (Jeol Ltd, Japan). The results confirm that activated water and combined plant extract additives may be used to improve the quality of fresh bread.

**Key words:** bread, bakery products, activated water, sweeteners, bound water, storage

## INTRODUCTION

An important issue in the manufacture of bread and baked goods is the consistency of the products. Very often, the ingredients used are quite dissimilar in terms of their properties, making it difficult to produce high-quality bread. For centuries, researchers have looked for ways to improve the quality of bread. Today, lactic acid bacteria (LAB), which produce a series of metabolites which have a positive effect on the texture of bread, are widely used in fermentation. Exopolysaccharides produced by LAB have the potential to replace more expensive hydrocolloids which are used as bread improvers (Arendt et al., 2007).

In addition to this method of influencing the dough's integral components, there is a growing trend which uses additives in the baking industry in order to achieve the optimum quality in terms of the technological properties of dough and the quality of the finished bread (Rosell et al., 2001). The interaction between sourdough and a number of additives such as exogenous enzymes and non-starch polysaccharides has been evaluated (Corsetti et al., 2000; Di Cagno et al., 2003). Researchers recommend the use of emulsifiers, hydrocolloids, and enzymes as additional methods to improve the quality of

bread. This improves the rheological properties of the dough and the quality of the finished bread, and stabilises its technological parameters (Sciarini et al., 2012).

Whatever improvement methods have been used by researchers (emulsifiers, enzymes, microbial ferments, complex enriching additive, influence on dough components, etc), everything ultimately depends on the dough's components, the interactions between those components, and their structure (McClements, 2007). The structure of the crumb is one of bread's fundamental quality characteristics. There is a direct relationship between the crumb structure, appearance, and the volume of finished products (Zghal et al., 1999), as well as their structure and texture (Pyler, 1988). Therefore we can conclude that a knowledge of bread structure makes it possible to exert an influence on its properties and quality.

## MATERIALS AND METHODS

### Bread ingredients

The raw materials for making bread and bakery products:

– wheat flour (gluten 28.5%, with an ash content of 0.55), produced by Grigorovich Bread Products Plant OJSC, Chelyabinsk City, Russia.

– activated water obtained using an ultrasonic processor, the USTA-0.4/22 OM (Volna, Russia), operating at a frequency of 22 ± 1.65 kHz and at 30% of maximum output power (400 W). The mechanism of ultrasonic cavitation in liquid systems occurs due to the formation of a high temperature and pressure shock waves (Naumenko & Kalinina, 2016). Physical effects include changes in viscosity, a dispersed state, and the strength of the colloidal system; chemical effects are linked, as a rule, to thermal mass exchange (Krasulya et al., 2015).

– a combined plant extract additive (CPEA) consisting of fucoidan and stevia derivative products (El'piner, 1963; Usov 2001; Shtrigul, 2009; Shestakov et al, 2013). Stevia is quite a well known natural sweetener, one which is recommended for diabetic nutrition. It can be used as a source of food in various forms – such as dried leaves and decoctions of those leaves, extracts, syrups or stevioside (a powder with stevia glycosides to be as purified as possible). Fucoidan is a sulphated heteropolysaccharide which is found in oceanic brown algae and in some echinoderms.

– a stevia solution prepared from stevioside powder (0.14% of flour weight), to which a calculated amount of 98 °C water was added and steeped for fifteen minutes. After steeping, the solution was filtered, cooled, and used at 35 °C. The stevioside infusion method eliminated the undesirable bitter aftertaste.

The main component of fucoidan molecules is the remains of sulfated α-L-fucose. Fucoidans are typically composed of other monosaccharides: galactose, mannose, xylose, uronic acids, and acetyl groups.

The bread was made using the recipes shown in Table 1. When using activated water, traditional bread recipes were used.

### Bread making

All of the samples that were studied were prepared using a straight dough process. A laboratory test was carried out on bread baking with a mass of 300 g and a temperature of 220 °C (Naumenko & Kalinina, 2016).

The raw materials underwent preliminary preparation. The flour was sifted and weighed on an automatic scale. Cake yeast was removed from its packaging. To ensure the even distribution of the yeast cells in the dough, the yeast was dissolved in water in yeast blending machines. The yeast suspension was prepared using one part yeast to two parts water at a temperature of 30 °C. Salt was dissolved before it was added. The water temperature did not exceed 40 °C.

**Table 1.** Dough recipes

| Ingredients, g | Bread | Reference sample | Bread with sugar completely replaced by fucoidan | Bread with sugar completely replaced by stevioside and fucoidan | Bread with sugar completely replaced by stevia syrup and fucoidan |
|---|---|---|---|---|---|
| Flour | 1,000 | 1,000 | 1,000 | 1,000 | 1,000 |
| CPEA, consisting of stevia and fucoidan derivative products | - | - | 1 | 1 | 1 |
| Vegetable oil | - | 140 | 140 | 140 | 140 |
| Sugar | - | 10 | - | - | - |
| Salt | 15 | 15 | 15 | 15 | 15 |
| Yeast | 20 | 20 | 20 | 20 | 20 |

The dough was mixed in one stage with all ingredients and the water according to the recipes. The dough's temperature after mixing was 31 ± 1 °C. During fermentation, the dough was punched down twice, at sixty minutes after the start of fermentation and again after two hours; the total duration of the dough fermentation process was 170 minutes.

The division of the dough for bread consisted of the following actions: the division of dough into pieces; its placing into pans; and the final proofing of product samples. The dough was divided into pieces using a dough-cutting machine.

The final proofing was carried out at room temperature (between 35–40 °C) and at a relative humidity level of between 70–75%. The proofing time for the formed pieces was sixty minutes.

The bread was cooled for three hours before being packed in plastic film, and then stored at a temperature of 2 ± 2 °C for 72 hours. Tests were carried out three and 72 hours after baking.

**Research methods**

The status of freshness was monitored, based on changes in organoleptic parameters (appearance, crust colour, crumb condition, taste, stickiness during mastication, and friability), and physical and chemical parameters (moisture content, swelling capacity, and friability).

The moisture content levels in the mass were determined by drying the test samples at a temperature of 130 °C for a period of forty minutes; the swelling capacity of the soft part of the bread was determined by the amount of water which was absorbed by this area of the bread within a period of five minutes (in terms of millilitres of water for each gram of dry matter (DM)); the friability, indicated as a percentage figure, was determined

according to the number of individual crumbs which were formed after shaking test samples of the soft part of the bread for fifteen minutes at a rotational speed of between 190–250 rpm.

Yeast activity was assessed using microscopy with an Altami-136T optical microscope (Altami, Russia). The microstructure was analysed using the microscopic studies that had been carried out with a Jeol JEM-2100 electron microscope (Jeol Ltd, Japan).

The baking of the bread and other bakery goods using various recipes, and studies of each product sample, were carried out in triplicate. A mathematical analysis was carried out on the results using generally accepted methods of statistical analysis, with the results being expressed as an arithmetical mean (M) and its standard deviation (m). Statistically significant differences amongst the groups were established using the Kruskal-Wallis criteria. In order to be able to detect statistically significant differences between two compared groups, the Manna-Whitney criteria (U) were used. Differences were deemed significant where $p < 0.05$. Statistical interconnections were studied using a non-parametric correlational analysis, calculating the coefficients of the correlation of rankings according to Spearman (Rs).

## RESULTS AND DISCUSSION

In order to evaluate the role of water in bread quality, samples were baked in which the dough was first prepared using tap water (the reference sample), and then using activated water.

The organoleptic evaluation of the studied samples suggests that the activated water and complex herbal supplements had a pronounced effect on the appearance, crust colour, crumb state, taste, stickiness during mastication, and friability.

Those samples which had been produced using activated water showed increased volume as well as a more developed, authentic, and thin-walled porosity, and high consumer appeal. Activated water influenced various quality indicators such as appearance, and the nature of the porosity, elasticity, and 'crumb chewability'. No significant changes in the flavour, aroma and/or colour of the products' crumbs were noted.

Those samples which were produced with the plant-extract additive can be characterised as having increased volume, a regular shape, and a slightly convex crust. A sufficiently uniform, thin-walled porosity with round-shaped pores enhances the customer appeal of the samples being studied, whilst the soft, elastic, and well-chewed crumb makes it even more attractive. It should also be noted that the bread samples which had been produced with sugar completely replaced by fucoidan were insufficiently sweet; those bread samples for which the sugar was completely replaced by stevioside and fucoidan had a sweet taste with a bitter aftertaste; and those bread samples for which sugar was completely replaced by stevia syrup and fucoidan had a typical, balanced taste.

When the physical and chemical parameters were checked, it was discovered that after the bread had cooled to room temperature (after three hours), the samples studied were almost the same in terms of moisture content, but different in terms of swelling capacity and friability (Table 2).

The friability of bread which had been produced with activated water was 0.7% less than that of the reference sample, and its swelling capacity was higher by 0.9 ml per 1g of DM. Differences between the values of the reference sample and those of bread which had been produced with activated water could be due to the intensified activity of the yeast and a more intensively developed protein matrix (Fig. 1).

**Table 2.** Changes in the physical and chemical parameters of the bread and bakery products samples during storage (3 hours)*

| Studied samples | Moisture content, % | Crumb porosity, % | Crumb acidity, % | Friability, % | Swelling capacity, ml $g^{-1}$ of DM |
|---|---|---|---|---|---|
| **Bread** | | | | | |
| Reference | 42.5 ± 0.1 | 73.0 ± 0.1 | 2.5 ± 0.2 | 5.6 ± 0.1 | 6.8 ± 0.2 |
| Using activated water | 43.2 ± 0.2 | 76.4 ± 0.3 | 2.9 ± 0.1 | 4.9 ± 0.2 | 7.7 ± 0.1 |
| **Bread with additives** | | | | | |
| Reference | 35.8 ± 0.2 | 74 ± 0.1 | 1.8 ± 0.2 | 4.4 ± 0.2 | 7.8 ± 0.2 |
| Bread with sugar completely replaced by fucoidan | 34.5 ± 0.1 | 83 ± 0.2 | 1.7 ± 0.2 | 3.7 ± 0.1 | 8.7 ± 0.2 |
| Bread with sugar completely replaced by stevioside and fucoidan | 37.6 ± 0.1 | 84 ± 0.1 | 1.6 ± 0.2 | 3.8 ± 0.1 | 9.1 ± 0.2 |
| Bread with sugar completely replaced by stevia and fucoidan syrup | 41.2 ± 0.2 | 82 ± 0.2 | 1.6 ± 0.2 | 3.9 ± 0.1 | 9.0 ± 0.2 |

*Results were obtained using generally accepted methods of statistical analysis and expressed as an arithmetical average and its standard deviation. Differences were deemed significant at $p < 0.05$

The structure of the samples is characterised by a large number of oval-shaped particles whose characteristics correspond to grains of starch. The grain surface is smooth and free of cracks, grooves, or pores. In the microstructure of the reference samples, equal proportions can be found both of large and fine grains of starch. The starch is present in the form of round or elliptical-shaped grains. Individual grains are slightly deformed.

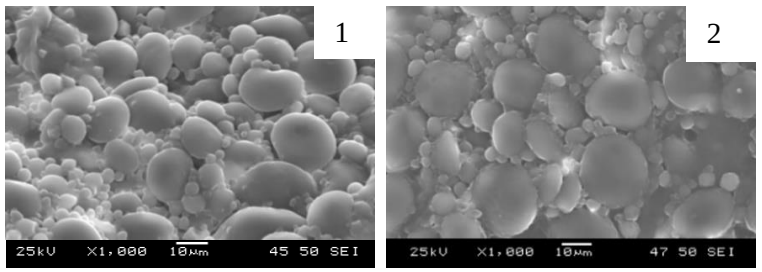



**Figure 1.** Dough microstructure: 1 – reference sample; 2 – sample using activated water.

Those samples which had been produced using activated water contain much fewer grains of starch. Swollen, significantly increased-size grains of starch predominate. The protein matrix is more distinct and developed. Some small starch grains have attached protein particles which make them angular. Large grains of starch have protein attached to them. Those protein particles which are attached to the fine grains of starch make them angular and faceted. Sometimes the protein matrix surrounds an entire group of starch grains in individual structural units. Fine starch grains, of which there are few, are interconnected by a protein web. The swollen protein matrix envelops the starch grains.

The bread with additives also differed positively from the reference samples. All of the test samples which had additives also had an increased crumb porosity of 8–10%. The crumb moisture mass fraction and the acidity of the test samples differed slightly. There was a decline in the friability of between 0.5–0.7% and an increase in the swelling index by 0.9–1.3 ml per 1g of DM. The CPEA which was added served to enhance the number of yeast cells, accelerating its maturation, and making the dough-making process more intensive as evidenced by the results of the study on the dough reference and test-sample microstructure (Fig. 2).

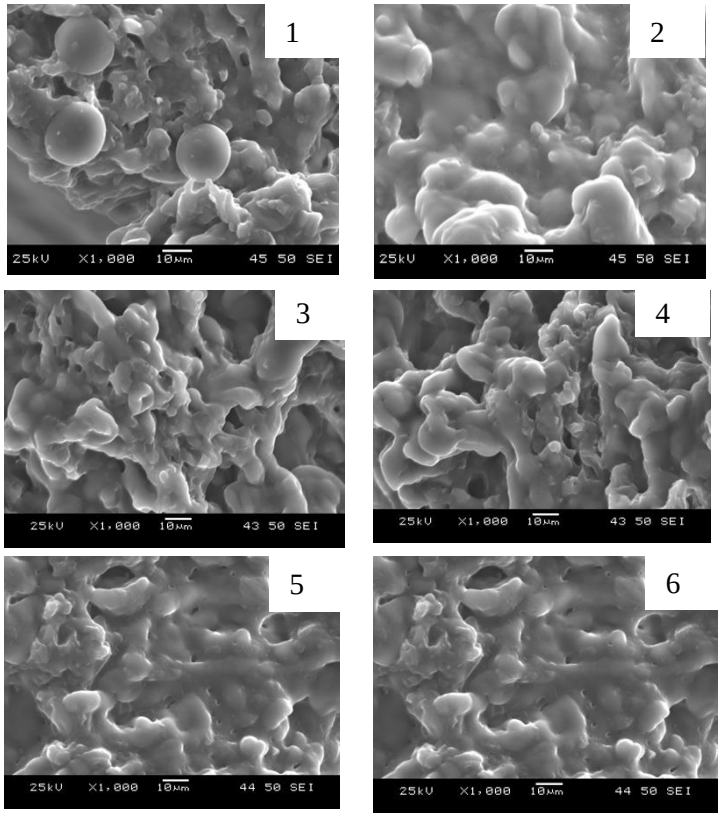



**Figure 2.** The microstructure of the bread crumb after 3 hours of storage: 1 – reference sample; 2 – sample using of activated water; 3 – reference for the bread with additives; 4 – bread with sugar completely replaced by fucoidan; 5 – bread with sugar completely replaced by stevioside and fucoidan; 6 – bread with sugar completely replaced by stevia syrup and fucoidan.

The baking process sets the sponge-like crumb texture in bread by creating a hierarchical structure of gas cells, from the macro to the micro scale within the bread crumb (Liu & Scanlon, 2003). The crumb structure in the test samples (bread made using activated water and samples with additives) is characterised by pores that are limited by the interporous walls which compose the spongy skeleton. A microscopic examination of the crumb's interporous walls shows that they consist of a solid mass of protein (gluten) which has coagulated in the baking process with the swollen, partially gelatinised starch grains embedded in it, as described in Rosell et al. (2001).

The starch grains in the pore walls are somewhat elongated, arranged parallel to their plane, and surrounded by a mass of coagulated protein. Only a few starch grains are in direct contact with each other, which is confirmed by other researchers (Semin et al., 2009). Protein coagulated substances form a spatially continuous phase of the bread crumb, and starch grains are only embedded in this system. This structure may be presented as a swollen, elastic jelly. The hard-to-distinguish, interporous walls consist of a solid mass of gluten (the protein coagulated in baking). In the test samples the entire surface of the starch grains is closely adjacent to the mass of coagulated protein, which means that no sharp, clearly visible boundary between them can be observed.

Results from the use of activated water and various ingredients (fucoidan, stevioside and fucoidan, and stevia and fucoidan syrup) were obtained from studying the bread samples after a period of 72 hours (Table 3).

Bread which was produced using activated water had significantly lower levels of friability than with the control sample, and the swelling capacity was significantly higher. The bread which was produced with additives also showed a positive deviation from the control samples. All test samples which included additives showed increased swelling capacity and a less pronounced intensity when it came to moisture loss in comparison to the control samples.

**Table 3.** Changes in the physical and chemical parameters of the bread and bakery products samples after storage (72 hours)

| Studied samples | Moisture content, % | Crumb porosity, % | Friability, % | Swelling capacity, ml $g^{-1}$ (d.m.) |
|---|---|---|---|---|
| **Bread** | | | | |
| Reference | 40.5 ± 0.2 | 66.0 ± 0.2 | 17.4 ± 0.1 | 3.2 ± 0.1 |
| Using activated water | 41.8* ± 0.2 | 71.0 ± 0.2 | 13.4* ± 0.2 | 4.5* ± 0.2 |
| **Bread with additives** | | | | |
| Reference | 34.6 ± 0.1 | 74.6 ± 0.2 | 12.8 ± 0.2 | 4.6 ± 0.2 |
| Bread with sugar completely replaced by fucoidan | 33.5* ± 0.2 | 76.2 ± 0.2 | 11.2* ± 0.1 | 6.7* ± 0.1 |
| Bread with sugar completely replaced by stevioside and fucoidan | 36.6* ± 0.1 | 78 ± 0.2 | 10.8* ± 0.1 | 7.1* ± 0.2 |
| Bread with sugar completely replaced by stevia syrup and fucoidan | 41.6* ± 0.1 | 78.2 ± 0.1 | 10.3* ± 0.1 | 7.3* ± 0.2 |

* significant differences ($p < 0.05$) in comparison to reference

All of the test samples showed an increased moisture content reading in comparison with the control samples, which could help to preserve the quality of stored bread. In

general, high water content in bread has been reported to increase its shelf life (Rogers et al., 1988; He & Hoseney, 1990) and to delay starch retrogradation (Andreu et al., 1999).

In the control samples, a partial transfer takes place from starch to a crystalline state, with an accompanying thickening of its structure. In the test samples (using activated water and the plant extract additives) this process occurs noticeably more slowly due to the formation of a more developed protein matrix during dough preparation. This also conforms to the data which was received in regard to changes to crumb friability and swelling capacity in baked goods during storage (Goryacheva et al., 1983; Schiraldi et al., 1996; Karim et al., 2000; Haros et al., 2002; Xie et al., 2004). The data which was obtained confirms well to the results from the study of dough microstructures in the control samples and test samples (Fig. 3).

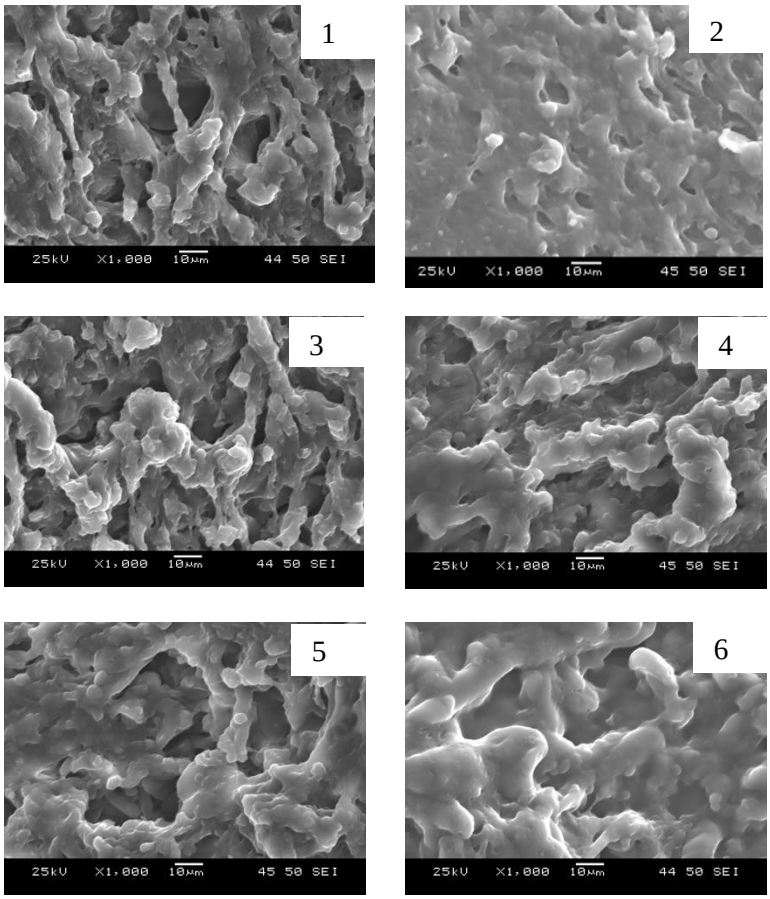



**Figure 3.** The microstructure of the bread crumb after 72 hours storage: 1 – control sample; 2 – sample using activated water; 3 – control for the bread with additives; 4 – bread with sugar completely replaced by fucoidan; 5 – bread with sugar completely replaced by stevioside and fucoidan; 6 – bread with sugar completely replaced by stevia syrup and fucoidan.

In the control sample's microstructure, interior layers of air can be clearly seen, which may indicate a decrease in the volume of starch granules in connection with the

formation of crystalline starch structures. The sample which used activated water has a more uniform, amorphous crumb structure, with a smaller number of interior air layers.

Also typical for the crumb structure of bread which has been made with fucoidan fully replacing sugar is the formation of pores that are limited by interporous walls which make up the spongy skeleton. The structures shown in those samples which had been produced using stevioside and with fucoidan fully replacing sugar, and in bread which had used stevia syrup with fucoidan fully replacing sugar, contain noticeable differences. For example, with the sample which was produced using stevioside and with fucoidan fully replacing sugar, it is possible to distinguish the starch granules and to determine the size of the pores. The microstructure of bread which has been produced with stevia syrup and with fucoidan fully replacing sugar can be described, as before, as a puffy, non-structured jelly with barely discernible interporous walls.

## CONCLUSIONS

The use of activated water enhances the accumulation of yeast cells, the development of protein matrix, and a more complete expansion of starch granules. Thanks to this, the bread has high consumer appeal, a beautiful appearance, increased volume, and a uniform, thin-walled porosity. The friability index significantly decreases, which also has a positive effect on the bread's organoleptic characteristics. Studies of the microstructure both of the dough and the finished product (following three hours of storage) confirm the organoleptic, physical, and chemical results. The test samples consist of a solid mass of protein (gluten) which has coagulated during baking, with swollen, partially gelatinised starch granules embedded in it. Those samples which were produced with activated water have even more greatly developed gluten.

The results of the study indicate a positive effect on the quality of the samples during storage (over the course of 72 hours). Bread which was produced with activated water had significantly lower levels of friability than did the control sample, and the swelling capacity was significantly higher. The resulting data is explained by the presence in those samples which were produced with activated water of a more uniform, amorphous crumb structure, with a decreased quantity of interior air layers. This is consistent with the data from Semin et al., (2009). This was also seen in other studies (Barcenas & Rosell, 2005; Brennan et al., 1996).

The use of various ingredients (fucoidan, stevioside, stevia syrup, and fucoidan) in bread production intensifies the dough-making process, makes the yeast cells accumulate more actively, and develops the protein matrix, which eventually has a positive effect on the quality of the finished product. All of those test samples which were produced with additives showed increased crumb porosity. A decrease in friability and an increase in swelling capacity were observed.

The results obtained were positive ones, and these were also maintained in the test samples during storage (over the course of 72 hours). The resulting data from a microscopic study of the crumb structure both from the control samples and the test samples leads one to the conclusion that changes in the microstructure of the control sample during storage occur with more intensity and, furthermore, an air layer forms around a portion of the pore surface which may result in the increased friability of the product. The full replacement of sugar with stevia syrup and fucoidan slows the staling

process most effectively, something that is confirmed by the less intensive changes in the microstructure.

ACKNOWLEDGEMENTS. This article was written with support from the government of the RF (Resolution No 211 of 16.03.2013), Agreement No 02.A03.21.0011, and subsidies for the fulfilment of a fundamental part of a state order, Project No 40.8095.2017/BCh. The work was also supported by Act 211 of the government of the Russian Federation, Contract No 02.A03.21.0011, and was carried out with the financial support of the UMNIK Programme's Assistance Fund.

## REFERENCES

Andreu, P., Collar, C. & Martı´nez-Anaya, M.A. 1999. Thermal properties of doughs formulated with enzymes and starters. *European Food Research and Technology* **209**, 286–293.

Arendt, E.K., Ryan, L.A.M., Dal Bello, F. 2007.Impact of sourdough on the texture of bread. *Food Microbiology* **24**, 165–174.

Barcenas, M.E. & Rosell, C.M. 2005. Effect of HPMC addition on the microstructure, quality and aging of wheat bread. *Food Hydrocolloids* **19**(6), 1037–1043.

Brennan, C.S., Blake, D.E., Ellis, P.R. & Schofield, J.D. (1996). Effects of guar galactomannan on wheat bread microstructure and on the in vitro and in vivo digestibility of starch in bread. *Journal of Cereal Science* **24**(2), 151–160.

Corsetti, A., Gobbetti, M., De Marco, B., Balestrieri, F., Paoletti, F., Russi, L. & Rossi, J. 2000. Combined effect of sourdough lactic acid bacteria and additives on bread firmness and staling. *J. Agric. Food Chem*. **48**, 3044–3051.

Di Cagno, R., De Angelis, M., Corsetti, A., Lavermicocca, P., Arnault, P., Tossut, P., Gallo, G. & Gobbetti, M. 2003. Interactions between sourdough lactic acid bacteria and exogenous enzymes, effects on the microbial kinetics of acidification and dough textural properties. *Food Microbiol*. **20**, 67–75.

El'piner, I.E. 1963. *Ultrasound. Physical, chemical, and biological effects.* Moscow. (in Russian).

Goryacheva, A.F. & Kuzminsky, R.V. 1983. Preservation of bread freshness. *Moscow. Publishing house: Light and food industry* 240 pp. (in Russian).

Haros, M., Rosell, C.M. & Benedito, C. 2002. Effect of different carbohydrases on fresh bread texture and bread staling. *European Food Research and Technology* **215**, 425–430.

He, H. & Hoseney, R. (1990). Changes in bread firmness and moisture during long term storage. *Cereal Chemistry* **67**, 603–605.

Karim, A.A., Norziah, M.H. & Seow, C.C. 2000. Methods for the study of starch retrogradation. *Food Chemistry* **71**, 9–36.

Krasulya, O., Bogush, V., Trishina, V., Potoroko, I., Khmelev, S., Sivashanmugam P. & Anandan S. 2015. Impact of acoustic cavitation on food emulsions. *Ultrasonics Sonochemistry* **30** 98–102.

Liu, Z. & Scanlon, M.G. (2003). Predicting mechanical properties of bread crumb. *Food and Bioproducts processing: Transactions of Institute of Chemical Engineers Part C* **81**(3), 224–238.

Naumenko, N.V. & Kalinina I.V., 2016 Sonochemistry effects influence on the adjustments of raw materials and finished goods properties in food production. *International Conference on Industrial Engineering, 19-20 May 2016, Chelyabinsk; Russian Federation* **870**, 691–696.

McClements, D.J. (2007). Understanding and controlling the microstructure of complex foods. *Cambridge: CRC Press*.

Pyler, E. J. (1988). Baking science and technology. *Kansas State: Sosland*. 850–910.

Rogers, D., Zeleznak, K., Lai, C. & Hoseney, R. (1988). Effect of native lipids, shortening, and bread oisture on bread firming. *Cereal Chemistry* **65**, 398–401.

Rosell, C.M., Rojas, J.A. & de Barber, B., 2001. Influence of hydrocolloids on dough rheology and bread quality. *Food Hydrocolloids* **15**, 75–81.

Schiraldi, A., Piazza, L. & Riva, M. 1996. Bread Staling: A Calorimetric Approach. *Cereal Chemistry* **73**(1). 32–39.

Sciarini, L.S., Ribotta, P.D., Leon, A.E. & Perez, G.T., 2012. Incorporation of several additives into gluten free breads: Effect on dough properties and bread quality. *Journal of Food Engineering* **111**, 590–597.

Semin, O., Gulum, S. & Serpil, S., 2009. The effects of gums on macro and micro-structure of breads baked in different ovens. *Food Hydrocolloids* **23**(8), 2182–2189.

Shestakov, S.D., Krasulya, O.N., Bogush, V.I. & Potoroko, I.Y. Technology and equipment for processing of food environment using cavitation disintegration. *Moscow, GIORD Publ*., 2013. 152 p. (in Russian).

Shtrigul', V.K. & Al'khamova G.K., 2009. Modern State and Prospects of Development of Food Industry and Catering. *Materials of II All-Russian Scientific and Practical Conference. Chelyabinsk*, South Ural St. Univ. Publ., pp. 158–159 (in Russian).

Usov, A.I., Smirnova, G.P. & Klochkova, N.G., 2001. Polysaccharide composition of certain brown algae of Kamchatka. *Bioorganic Chemistry* **27**(6), 444–448 (in Russian).

Xie, F., Dowell, F.E. & Sun, X.S. 2004. Using visible and near-infrared reflectance spectroscopy and differential scanning calorimetry to study starch, protein, and temperature effects on bread staling. *Cereal Chemistry* **81**(2), 249–254.

Zghal, M.C., Scanlon, M.G. & Sapirstein, H.D., 1999. Prediction of bread crumb density by digital image analysis. *Cereal Chemistry* **76**(5), 734–742.

*Agronomy Research* **15**(S2), 1386–1398, 2017

# A study of the forms of bound water in bread and bakery products using differential thermal analysis

L. Nilova[1], N. Naumenko[2*] and I. Kalinina[2]

[1]Peter the Great St. Petersburg Polytechnic University, Institute of Industrial Management, Economics and Trade, Graduate School of Commodity and Service, Novorossiyskaya Street 50, 194021, Saint-Petersburg, Russia;
[2]South Ural State University, Higher School of Medicine and Biology, Department of Food and Biotechnology, 85 Lenina Avenue, 454080, Chelyabinsk, Russia;
[*2]South Ural State University, Higher School of Medicine and Biology, Department of Food and Biotechnology, 85 Lenina Avenue, 454080, Chelyabinsk, Russia;
* Correspondence: naumenko_natalya@mail.ru

**Abstract.** The objective is to study the forms of bound water in bread and bakery products using differential thermal analysis, changes to these forms corresponding to different recipe components, and changes occurring during storage. The subject of this research are bread and bakery products made of wheat flour (with gluten content of 28.5%, and ash content of 0.55%): without added fat; with tap water or activated water used for dough mixing; with varying fat content (4 and 14%); protein-enriched with cedar nut flour (5%); and dietary (food) fiber-enriched with red-fruited mountain ash and sea buckthorn powder (5%). The reference samples of bread and bakery products were stored in plastic film bags at 20 ± 2 °C for a period of 72 hours. The freshness was monitored by changes in the physical-chemical parameters (moisture content, swelling capacity, friability). The various forms of bound water were determined using the method of differential thermal analysis on a simultaneous TGA-DTA/DSC thermogravimetric analyzer, with a programmable temperature regime. Based on the obtained digital data on thermogram (TG) change, using Pearson's criterion, a mathematical model has been created to identify the linear sections with a different inclination angle which are characterized by a constant rate of water removal. For all studied samples of bakery products, 6 linear sections were identified, but statistically significant results were obtained for sections III, IV and V, with the exception of section III for bakery products with cedar flour. Use of activated water, fat, and additives of cedar flour, powders of red-fruited mountain ash and sea-buckthorn in the production of bread and bakery products leads to redistribution of water forms, which is confirmed by changes in the boundaries of the linear sections, both for freshly made products and for products after storage. As a result, these products stay fresh longer.

**Key words:** bread, bakery products, activated water, powder, bound water, storage

## INTRODUCTION

One of the main problems of the bread-baking industry is the limited storage time of the products, i.e. of bread and bakery products. Despite the fact that bread and bakery products are usually packed individually, its usual storage time is still less than 72 hours, due to the desiccation and staling of the products. For more than 160 years, scholars have

investigated the processes of staling, but no universally recognized theory has been generated so far. Most scholars consider starch retrogradation to be the main cause of staling. Over time, the starch, gelatinized in baking, releases the water that it has absorbed, and thus the liberated hydroxyl groups of glucose residue are bound with their hydrogen bonds. Partial transition of the starch into its crystalline state occurs there, accompanied by its structural densification. This is what is a cause of changes in friability and swelling capacity of the crumb of the bread and bakery produce during storage (Goryacheva et al., 1983; Schiraldi et al., 1996; Karim et al., 2000; Haros et al., 2002; Xie et al., 2004; Cocchi et al., 2005). Slowing-down of the process of retrogradation is facilitated by both physical factors (kneading of dough, temperature pattern of baking, storage conditions and freezing of partially baked bread) and other factors which enable the separation of starch grains and prevent starch aggregation. (Rasmussen & Hansen, 2001; Fessas & Schiraldi, 2001; Bárcenas et al., 2003; Azizi & Rao, 2005; Fessas & Schiraldi, 2005; Le-Bail et al., 2011; Bosmans et al., 2013; Eckardt et al., 2013; Fadda et al., 2014; Al-Hajji et al., 2016). As the quantity of proteins and other biopolymers grows the process of staling starts to slow down (Goryacheva et al., 1983; Xie et al., 2004; Pashchenko & Zharkova, 2006**)**. The reason is believed to be the complicated redistribution of water depending on the bonds between the biopolymers of bread. This question remains unexplored and controversial.

In bread and bakery products, water is present in free and bound forms (Goryacheva et al., 1983; Pashchenko & Zharkova, 2006; Palyvoda et al., 2013). The amount of easily removable water in these products cannot be high because of the predominance of biopolymers - proteins and starch, and non-starch polysaccharides. Part of the water can easily penetrate into micro pores in the protein and be retained by a macromolecular matrix (Damodaran et al., 2007; Nechayev, 2015); some authors refer to this form of water as physic-mechanically bound (Yurchak et al., 1988). Low energy binding is characteristic of osmotically retained water. Adsorbed water (water of polymolecular and monomolecular layer) and organically bound water is considered as bound water. Adsorption is accompanied by a thermal effect, and removal of this water requires a large amount of energy. The stronger the bond, the greater the energy required to break it (Duckworth, 1980; Yurchak et al., 1988; Damodaran et al., 2007; Nechayev, 2015).

Differential thermal analysis makes it possible to define physical and chemical changes of substance using a programmed temperature increase. In the course of the analysis, a gradual break of water molecules bonds with biopolymers of bread and bakery products takes place, accompanied by a change in mass of the sample. Previously, differential thermal analysis could define only the total amount of free and bound water (Schiraldi et al., 1996); now, bound water is considered as aggregate of water forms having different binding energy. In the works (Antipov et al., 2010; Ostrikov & Napolskikh, 2012; Palyvoda et al., 2013; Kazantseva, 2015) researchers were able to determine up to 5 sections of stepwise behavior of water in food systems with different binding energy by using differential thermal analysis. Thermogravimetric analyzers equipped with analog-to-digital converters simplify the process of inflection points defining and identification of linear sections of stepwise water removal from food systems, corresponding to different forms of water binding.

The objective is to study the forms of bound water in bread and bakery products using differential thermal analysis, and to study their changes depending on the formula components and during storage.

## MATERIALS AND METHODS

### Bread ingredients

The materials to make bread and bakery products:

- Wheat flour (28.5% of gluten, and 0.55% ash content) made by Kombinat khleboproductov imeni Grigorovicha, OAO (A.F. Grigorovich Bread and Bakery Plant OJSC), Chelyabinsk, Russia;

- Activated water (catholyte) obtained by electrolysis (processing time 30 minutes at 200 V) using AP-1 device made by *Aquapribor* Research and Development Company in the Republic of Belarus. Electrochemical activation of water leads to the rupture of hydrogen bonds in the $[H_2O]_x$ associates and to the increased concentration of $H_2O$ monomolecules (Naumenko, 2014);

- *Sloboda* refined deodorized sunflower oil, produced by *Efko* OAO (OJSC), Russia;

- Cedar nut flour containing 34% proteins (Nilova et al., 2013), produced by *Spetsialist* OOO (*Specialist* LLC), Russia;

- The powders of red-fruited mountain ash (domestic sp.) and sea buckthorn (*Vitaminnaya* breed) were obtained from squeezed berries, dried at 50 to 55 °C (to a moisture level of 6%) and milled into powder.

Bread and bakery products were made using the formulas shown in Table 1 (Nilova, 2012). For products using activated water, traditional bread making recipes were used.

**Table 1.** Dough recipes

| Ingredient, g | Bread | Bakery products with additives | | | | |
|---|---|---|---|---|---|---|
| | | 4% fat | 14% fat | cedar nut flour | red-fruited mountain ash powder | sea buckthorn powder |
| Flour | 1,000 | 1,000 | 1,000 | 950 | 950 | 950 |
| Cedar nut flour | - | - | - | 50 | - | - |
| Red-fruited mountain ash powder | - | - | - | - | 50 | - |
| Sea buckthorn powder | - | - | - | - | - | 50 |
| Vegetable oil | - | 40 | 140 | 40 | 140 | 140 |
| Sugar | - | 50 | 140 | 50 | 140 | 140 |
| Salt | 15 | 15 | 15 | 15 | 15 | 15 |
| Yeast | 20 | 20 | 20 | 20 | 20 | 20 |

### Bread making

All reference samples were produced using a straight dough method. The experimental laboratory baking of the 300 g bread loaves and 100 g bakery products was done at 220 °C (Nilova et al., 2013; Naumenko, 2014; Kalinina, 2014).

Then the reference samples were cooled for 3 hours, and packed in plastic film bags and stored at a temperature of 20 ± 2 °C for 72 hours. The tests were performed 3 and 72 hours after baking.

**Research Methods**

The freshness condition was assessed based on changes in the physical and chemical properties, such as the moisture content, swelling capacity and friability.

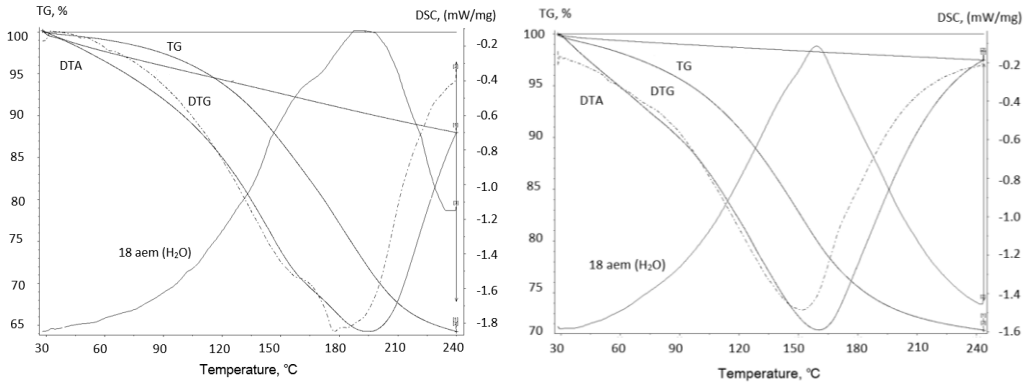
Moisture content was determined by drying the reference samples at 130 °C for 40 minutes; the swelling capacity of the crumb was determined by the quantity of water absorbed by the crumb in 5 minutes (as milliliters per 1g of dry matter) within 5 minutes; the friability was determined according to the quantity of crumbs formed in 15 minutes as a result of hanging and shaking crumb pieces at 190–250 rpm (as percentage of crumbs formed relative to the remaining soft part).

Forms of water binding in bread and bakery products were determined by means of differential thermal analysis using a MOM manufactured simultaneous TG-DTA / DSC thermogravimetric analyzer, a Paulik Erdey system model, made in Hungary. Studies were carried out in quartz crucibles with sample weight of 1g. $Al_2O_3$ calcinated at temperature of 1,800 °C was used as a reference substance. The oven heating temperature variation rate was 2.5 °C $min^{-1}$, and the maximum heating temperature was 220 °C. Using the analog-to-digital converter, we obtained curves in digital form. In order to determine the temperature ranges in relation to water evaporation in various forms of binding, we used the TG curve, reflecting the change in sample weight in relation to temperature or time. In order to identify forms of water binding we used the piecewise-linear approximation method. To define temperature intervals corresponding to water evaporation for various bound forms of water molecules, TG curves characterizing sample mass change versus temperature were used. Using Pearson's criteria, a piecewise-line approximation model for identification of water molecules bound forms was created.

All TG curves obtained for the samples of bread and bakery products show similar behavior (Fig. 1), but size of the temperature zones corresponding to evaporation of water having different bonds energy were different. In order to construct a model for the TG curves, conversion values α were calculated and curves -lgα versus 1,000 $T^{-1}$ were plotted using a piecewise-linear approximation Y = F (X). For each of the curves obtained, 6 linear sections with different inclination angles to the horizontal axis were identified (Fig. 2). The model is described by the linear function $Y_i=A_i \cdot x+B_i$ and $X_i< x \leq X_{i+1}$, defining their definition domains. The model parameters subject to identification are the coefficients Ai, Bi, i = 1 ... N and section areas Xi, i = 1 ... N-1, where N is the number of linear sections.

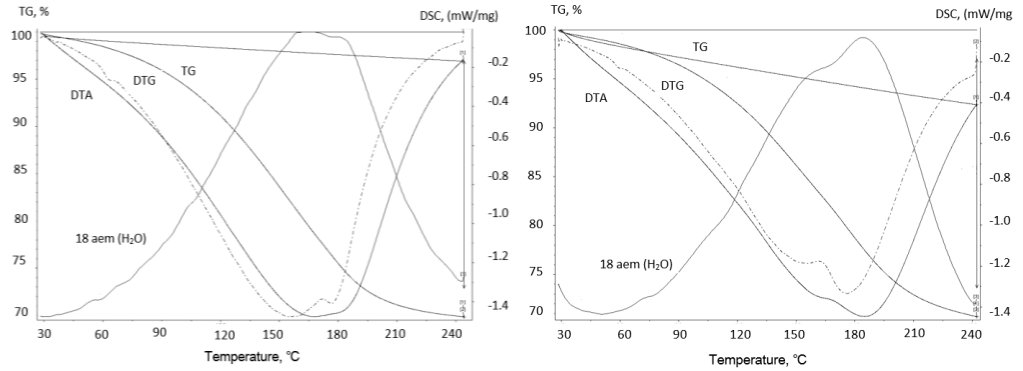
Rate of weight change α was calculated as a ratio of weight to total amount of water contained in the product which was defined by TG curve at the end of dehydration process. The form of the resulted curve reflects complicated nature of water and dry matter interaction in the products and assumes difference in the rate of water release for the different sections of this curve.

3 hours after baking




Bread 14% fat bakery products

72 hours after baking




Bread 14% fat bakery products

**Figure 1.** Thermogram of the reference samples of bread and bakery products, 3 (above) and 72 (below) hours after baking: TG – sample mass, DTA – temperature change rate, DTG – weight change rate.

Therefore, curves of the matter transformation ratio versus temperature allow studying of various, kinetically unequal forms of moisture bounding and reflects difference in dehydration rate (Ostrikov & Napolskikh, 2012).

To obtain dehumidification mechanism data on the basis of the obtained curves, to define temperature range and amount of moisture desorbed with approximately equal rate, this curve in coordinates ($-\lg\alpha$; 1,000/T) was used.

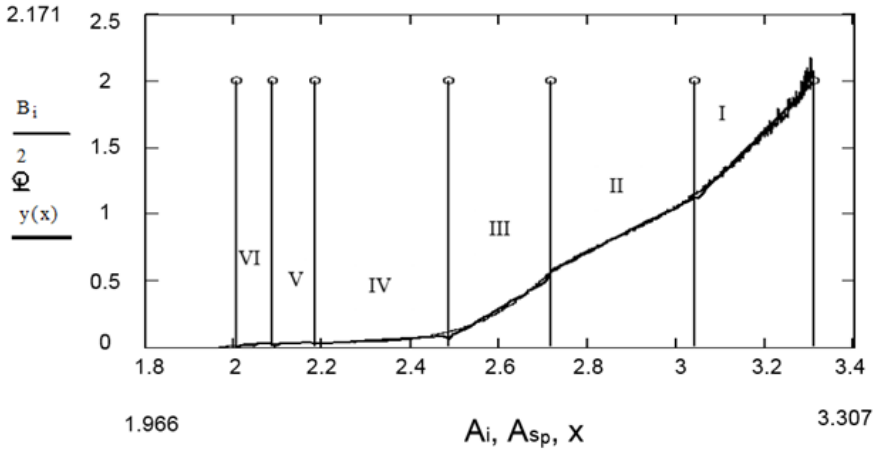



**Figure 2.** The -lgα to 1,000 $T^{-1}$ ratio piecewise-linear approximation model.

The baking of the bread and bakery products using various recipes, and the study of each sample were carried out three times. The mathematical results were calculating using conventional statistics methods, and were expressed as arithmetic mean (M) and standard deviation (m). In order to identify statistically significant differences between two compared groups, the Mann-Whitney criteria (U) was used. Differences were considered significant where $p < 0.05$.

## RESULTS AND DISCUSSION

To assess the role of water in the bread staling process, samples were baked in which the dough mixing was carried out first using tap water (control) and then using activated water (catholyte). After cooling the bread to room temperature (3 hours), the reference samples had almost the same moisture content values, but different swelling and friability values (Table 2). The friability of the bread with activated water was 0.7% less than that of control sample, and its swelling capacity was higher by 0.8 ml per 1 g of dry weight. Differences between the parameters of the control samples and the bread made with activated water could be associated with the redistribution of the bound water forms in the bread (Fig. 3).

Bread sample thermograms showed similar behavior, but by creating a piecewise-linear approximation model and using Pearson's criterion, it was possible to identify 6 linear sections with a different inclination angle. The curves for bread (reference sample) and for bread with activated water have different linear sections boundaries and only the boundaries of sections II, III, IV, and V are statistically significant. This makes it possible to expect a quantitative difference in the content of different forms of water (Fig. 3).

**Table 2.** Changes in the physical and chemical parameters of the bread and bakery product samples during storage *

| Reference samples | Moisture content, % | | Friability, % | | Swelling ml $g^{-1}$ (of d.m.) | |
|---|---|---|---|---|---|---|
| | after 3 hours | after 72 hours | after 3 hours | after 72 hours | after 3 hours | after 72 hours |
| **Bread** | | | | | | |
| Reference sample | 42.0 ± 0.3 | 40.5 ± 0.4 | 5.5 ± 0.2 | 17.4 ± 0.2 | 6.7 ± 0.2 | 3.2 ± 0.1 |
| Sample with activated water (catholyte) | 42.5 ± 0.2 | 41.3 ± 0.3 | 4.8 ± 0.1 | 13.6 ± 0.3 | 7.5 ± 0.1 | 4.2 ± 0.1 |
| **Bakery products** | | | | | | |
| 4% fat | 39.2 ± 0.4 | 38.3 ± 0.2 | 4.3 ± 0.1 | 12.5 ± 0.3 | 7.7 ± 0.1 | 5.1 ± 0.1 |
| With cedar nut flour | 39.8 ± 0.1 | 39.2 ± 0.2 | 3.7 ± 0.1 | 9.7 ± 0.1 | 8.7 ± 0.1 | 6.9 ± 0.1 |
| 14% fat | 35.5 ± 0.2 | 34.8 ± 0.1 | 4.0 ± 0.1 | 11.2 ± 0.2 | 8.5 ± 0.1 | 6.1 ± 0.1 |
| With red-fruited mountain ash powder | 34.7 ± 0.1 | 34.2 ± 0.1 | 3.1 ± 0.1 | 8.1 ± 0.1 | 9.3 ± 0.1 | 7.5 ± 0.1 |
| With sea buckthorn powder | 34.5 ± 0.1 | 34.0 ± 0.1 | 2.8 ± 0.1 | 6.7 ± 0.1 | 8.8 ± 0.1 | 6.7 ± 0.1 |

*Results were obtained using generally accepted methods of statistical analysis and expressed as an arithmetical average and its standard deviation. Statistically significant by Mann-Whitney criterion $p < 0.05$.

For bread (reference sample) the first dehydration stage (linear section I) was observed at the temperature range of 26.2–56.1 °C, apparently caused by the removal of free water, of which there was a small amount. In linear section II (56.1–91.3 °C) there was maximal water loss – almost half of all the water removed in the course of differential thermal analysis (DTA). This could be caused by the beginning of the gelatinization of the starch, elimination of water contained in the macromolecular matrix, and water bound with the starch hydration centers (Goryacheva et al., 1983, Pashchenko & Zharkova, 2006, Palyvoda et al., 2013, Kazantseva, 2015). In the linear section III (91.3–125.8 °C) the amount of removed water decreased in comparison with linear section II – by 38.3%, but was significant – 31.1% of the total amount of water removed at DTA. Perhaps the complete gelatinization of the starch contributed to the release of the hydration shell of hydrophilic groups of the starch polymer and polypeptide chains (Pashchenko & Zharkova, 2006). The further temperature rise in linear section IV (125.8–180.6 °C) could result in denaturation of the protein molecules and release of the bound water portion contained in the closed cells of protein micelles (Goryacheva et al., 1983, Palyvoda et al., 2013, Kazantseva 2015). However, since the protein content of the bread is less than that in starch, the proportion of water removed in this section was only 7.4%. The last two linear sections – V (180.6–201.2 °C) and VI (201.2–225 °C) - are probably showing the difficult-to-remove, tightly bound water – a 'monolayer' strongly interacting with hydrophilic groups of nonaqueous biopolymers), as well as crystalline hydrates (Nechayev, 2015). But the amount of water removed in these sections differed by 1.5x. Thus, statistically significant values were obtained only for curve section V.

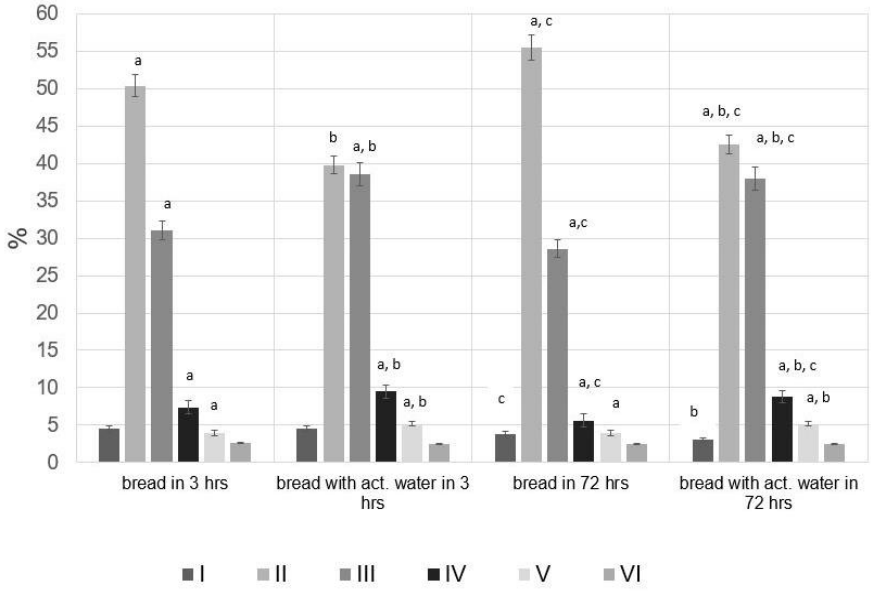



**Figure 3.** Changes in the forms of bound water in the studied samples of bread during storage. Statistically significant by Mann-Whitney criterion ($p < 0.05$) differences between the line sections: a – in bread samples; b – in bread with activated water in comparison with reference sample; c – in bread samples during storage.

For bread with activated water, statistically significant lengths were identified for linear curve sections III, IV and V. A statistically significant widening of these sections and a narrowing of section III were observed. Redistribution of the forms of bound water may be associated with the fuller swelling of the proteins as a result of the penetration of activated water into its structure in the form of monomolecules (Yurchak et al., 1988, Naumenko, 2014).

After 72 hours of storage, water redistribution occurred in all bread samples. The general trends in the changes to the linear sections were similar irrespective of the kind of water used for bread making, but the linear sections typical for the removed water differed in size. After storage, a statistically significant widening was observed only for linear curve section II: for reference sample - by 5.1%; for bread with activated water - by 2.7%. The amount of removed water for sections III and IV showed a statistically significantly decrease of 2.5 and 1.8%, respectively, in comparison with the reference sample, and by 0.5 and 0.7%, respectively, in comparison with the bread with activated water. The boundaries of linear sections V and VI remained unchanged. Only in the reference sample was there a significant decrease in linear section I. Changes in the forms of bound water in bread during storage could be associated with aging of biopolymers. The result is that they become denser, forming cracks between the starch and protein, which leads to increased friability of the bread and reduces its swelling capacity (Table 2). As a result, the removed water content in the bread increases in linear section II, and it decreases in section I due to the shrinkage/desiccation of the products that is confirmed by the changes in the moisture content values. In the bread with

activated water the process is slower - the moisture content after 72 hours is 0.8% less in comparison to the control sample.

When fats and sugar are used in the recipes of the bakery products, less water is required for the dough mixing, so the initial moisture content of the products is lower in comparison to the bread. Fats facilitate the plasticization of the dough, adsorbing the hydrophobic part of the molecule on the surface of the starch grains, and increase the quantity of hydrophilic parts (Goryacheva et al., 1983). Therefore, the swelling capacity of bakery products depends on the amount of added fat: buns with 14% fat have a higher value than buns with 4% fat (Table 2). Increasing the elasticity of the dough by enveloping the starch grains with fats reduces the friability of bakery products when cooled to room temperature for 3 hours. But increasing the amount of fat from 4 to 14% has no significant effect on friability values, which change only by 0.3%. The quantity of added fats in the recipes of bakery products also had no significant effect on the redistribution of forms of bound water (Figs 4 and 5)., Bakery products with 4 and 14% fat content showed statistically significant differences in the sizes of linear sections II, III and IV.. Linear sections III and IV had statistically significant increases in size - by 0.5% and 1.2%, respectively. And linear section II decreased by 1.7%. In comparison with reference sample (bread), the addition of fat to the recipe caused statistically significant changes in the sizes of sections II, III, V, and for bakery products with 14% of fat, in the size of section IV.

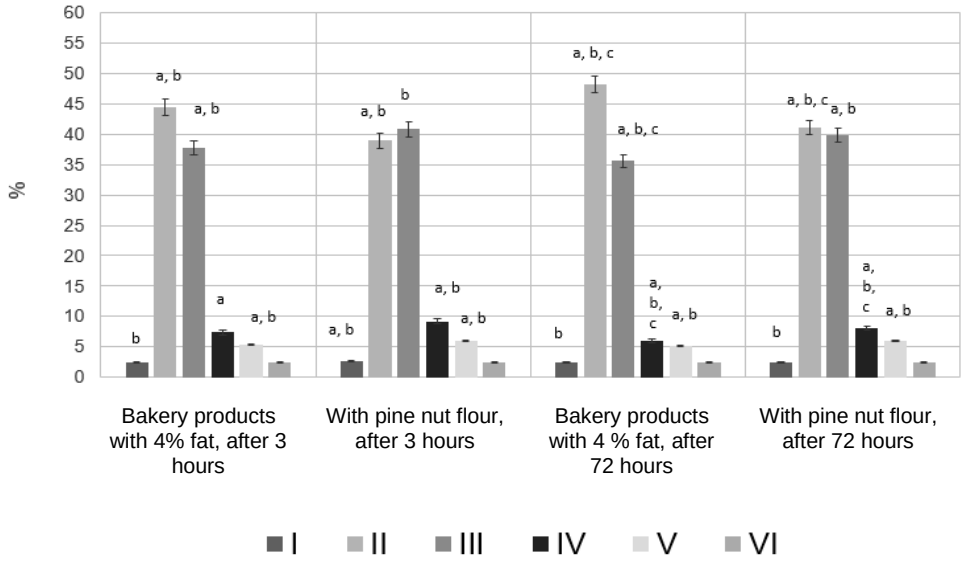



**Figure 4.** Changes in the forms of bound water in the studied samples of bakery products with 4% fat during storage. Statistically significant by Mann-Whitney criterion ($p < 0.05$) differences between the line sections: a – in bakery product samples; b – in bakery product in comparison with reference sample; c – in bakery product samples during storage.

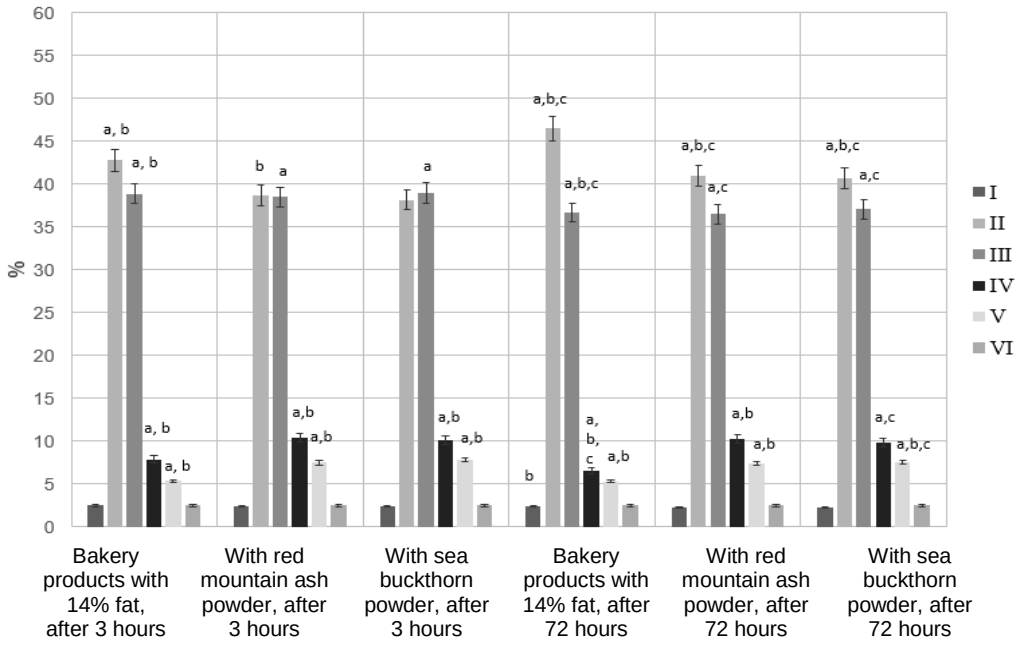



**Figure 5**. Changes in the forms of bound water in the studied samples of bakery products with 14% fat during storage. Statistically significant by Mann-Whitney criterion ($p < 0.05$) differences between the line sections: a – in bakery product samples; b – in bakery product in comparison with reference sample; c – in bakery product samples during storage.

Such a change in the size of linear sections for the bakery products in comparison with the bread was caused by the staling processes, despite a 3 time difference in mass. And if the crumb swelling capacity after 72 hours decreased by 2 times, then swelling capacity of the bakery products with 4% fat decreased by 1.5 times and bakery products with 14% fat - by 1.4%. A reverse behavior was observed in the changes of the crumb friability values. After 72 hours the bread friability increased by 3.1 times, and bakery products friability – by 2.9 times and friability of products with 4% and 14% fat – by 2.8 times, respectively. Such changes may be associated with the blocking with fats of crumb starch aggregation during storage, enveloping with thin films not only the starch but also the protein. As a result, they released water more slowly as compared to bread, thus preventing their densifying and formation of air layers. The amount of water in linear section III decreased by 5.6% and 5.7%, and in section IV by 17.6% and 16.5%, respectively for bakery products with 4% and 14% fat (Figs 4 and 5).

Cedar nut flour (Table 1) was used to enrich the bakery products with proteins by replacing 5% of the wheat flour in the recipes with 4% fat. Bakery products with 4% fat served as a reference sample. Already 3 hours after baking, the freshness parameters of the bakery products with cedar nut flour differed from those of the reference sample: swelling capacity was 11.5% higher, and the friability was 16% lower (Table 2). The protein in the cedar nut flour influenced the forms of bound water that mainly led to increase in the amount of water removed in linear section III in comparison to the reference sample. Changes in the amount of water removed in sections IV and V also increased, but these additions were less appreciable. The increased amount of bound

water slowed down the staling processes. After 72 hours, bakery products with cedar flour had lower values of friability and swelling capacity. Redistribution of forms of bound water in the crumb of the bakery products was slower, especially in section III. This is reflected in the smaller loss of moisture content in the product after storage. It should be noted that there were no changes in the amount of removed water in linear sections V and VI.

Pomace of red-fruited mountain ash and sea-buckthorn powders were used in the recipes of bakery products with 14% fat by replacing 5% of the wheat flour. As these products comparison, bakery products with 14% fat without additives were used as a reference sample. After 3 hours the bakery products with the powders had lower values of moisture content than the reference sample, likely due to the strong water binding in linear section V formed on the surface of the fiber and pectin with a high degree of esterification (Nilova, 2012). Also, the amount of removed water for linear section IV showed a statistically significant increase, and to a greater degree than for linear section III. Change in boundaries of linear section III were not statistically significant for bakery products with powder in comparison with the reference sample. Nevertheless, bakery products with powder had a lower friability value and greater swelling capacity in comparison with the reference sample. Water redistribution during storage of the bakery products with powder was insignificant, particularly for linear sections IV and V, which is confirmed by the absence of statistically significant changes. Only for bakery products with sea-buckthorn did changes in the boundaries of linear section V during storage reach statistically significant values. The amount of removed water for linear section III increased to a greater degree – by 5%, irrespective of the kind of powder, which insignificantly increased the water ratio for linear section II. The change in these boundaries during storage was statistically significant. As a result, after 72 hours the bakery products with powder were absolutely fresh, as confirmed by measurements of their physical and chemical parameters.

## CONCLUSIONS

Based on results of differential thermal analysis carried out using a Simultaneous TGA-DTA/DSC thermogravimetric analyzer, Paulik Erdey system model with analog to digital converter, which is able to define the sample mass change versus temperature (TG) in digital form, and using Pearson's criterion, a mathematical model was created to identify the linear sections with a different inclination angle which are characterized by a constant rate of water removal. For all studied samples of bakery products, 6 linear sections were identified, but statistically significant results were obtained for sections III, IV and V sections, with the exception of section III for bakery products with cedar flour. In order to find correspondence between the identified sections and different forms of bound water, it is necessary to continue this study using other physical methods.

The importance of water in the bread staling process is confirmed by the results of activated water use for dough mixing. Activation of water, which causes an increased concentration of $H_2O$ monomolecules, leads to deeper penetration of water molecules into the structure and a fuller swelling of the biopolymer. In freshly baked bread with activated water there is a statistically significantly increase in the boundaries of linear sections III, IV and V. As a result, bread with activated water loses water slower, which

is confirmed by statistically significant changes in the sizes of linear sections II. III. IV during storage in comparison with the reference sample.

The use of various ingredients (fat, cedar flour, powders of red-fruited mountain ash and sea-buckthorn) in the manufacture of bakery products leads to redistribution of water forms both in freshly made products, and in products after storage. Linear sections statistically significantly increase for in freshly baked products, there is a statistically significant increase in linear section III when using fat and cedar flour, and in sections IV and V when using powders of red-fruited mountain ash and sea-buckthorn. As a result, less water is lost from these products, and the staling process is slowed, which is confirmed by the results of the study of the mass moisture ratio, friability and swelling capacity of the bakery products.

ACKNOWLEDGEMENTS. This article was written with support from the Government of the RF (Resolution №211 of 16.03.2013), Agreement № 02.A03.21.0011 and subsidies for the fulfillment of a fundamental part of a state order, project №40.8095.2017/BCh. The work was supported by Act 211 Government of the Russian Federation, contract № 02.A03.21.0011.

## REFERENCES

Al-Hajji, L., Nassehi, V. & Stapley, A. 2016. Spatial variation of starch retrogradation in Arabic flat bread during storage. *Journal of Food Engineering* **187**, 44–52.

Antipov, S.T., Zhuravlev, A.V., Kuznetsova, I.V., Chernousov, I.M. & Baranov, A.Y. 2010. Research of the forms of binding moisture in seed of amaranth varieties by differential thermal analysis. *Storage and Processing of Farm Products* **8**, 14–16 (in Russian, English abstr.).

Azizi, M.H. & Rao, G.V. 2005. Effect of storage of surfactant gels on the bread making quality of wheat flour. *Food Chemistry* **89**, 133–138.

Bárcenas, M.E., Haros, M., Benedito, C. & Rosell, C.M. 2003. Effect of freezing and frozen storage on the staling of part-baked bread. *Food Research International* **36**, 863–869.

Bosmans, G.M., Lagrain, B., Fierens, E. & Delcour, J.A. 2013. The impact of baking time and bread storage temperature on bread crumb properties. *Food Chemistry* **141**, 3301–3308.

Cocchi, M., Foca, G., Marchetti, A., Sighinolfi, S, Tassi, L. & Ulrici, A. 2005. Use of multivariate analysis of MIR spectra to study bread staling. *Annali di Chimica* **95**, 657–666.

Damodaran, S., Parkin K.L. & Fennema O.R. 2007. *Fennema's Food Chemistry, Fourth Edition.* CRC Press. Taylor & Francis Group. Boca Raton. London, New York. 553 pp.

Duckworth, R.B. 1980. Water relations of foods. *Publishing House 'Food Industry',* Moscow. 376 pp. (in Russian).

Eckardt, J., Ohgren, C., Alp, A., Ekman, S., Astrom, A., Chen, G., Swenson, J., Johansson, D. & Langton, M. 2013. Long-term frozen storage of wheat bread and dough - effect of time, temperature and fibre on sensory quality, microstructure and state of water. *Journal of Cereal Science* **57**, 125–133.

Fadda, C., Sanguinetti, A.M., Del Caro, A., Collar, C. & Piga, A. 2014. Bread staling: updating the view. *Comprehensive reviews of Food Science and Food Safety* **13(**4), 473**–**492.

Fessas, D. & Schiraldi, A. 2001. Water properties in wheat .our dough I: Classical thermogravimetry approach. *Food Chemistry* **72**, 237–244.

Fessas, D. & Schiraldi A. 2005. Water properties in wheat flour dough II: classical and Knudsen thermogravimetry approach. *Food Chemistry* **90**, 61–68.

Goryacheva, A.F. & Kuzminsky, R.V. 1983. Preservation of bread freshness. Moscow. *Publishing house: Light and food industry*. 240 pp. (in Russian)

Haros, M., Rosell, C.M. & Benedito, C. 2002. Effect of different carbohydrases on fresh bread texture and bread staling. *European Food Research and Technology* **215**, 425–430.
Karim, A.A., Norziah, M.H. & Seow, C.C. 2000. Methods for the study of starch retrogradation. *Food Chemistry* **71**, 9–36.
Kazantseva, I.L. 2015. Research of forms of a moisture communication in the raw materials and the finished confectionery by thermal analysis method. *Storage and Processing of Farm Products* **7**, 36–40 (in Russian. English abstr.).
Kalinina, I.V. 2014. Innovative approaches to perfecting manufacturing technologies of bread and bakery products. Publishing house of SUSU, Chelyabinsk, 160 pp. (in Russian).
Le-Bail, A., Leray, G., Perronnet, A. & Roelens, G. 2011. Impact of the chilling conditions on the kinetics of staling of bread. *Journal of Cereal Science* **54**, 13–19.
Naumenko, N.V. 2014. Physical nature factors in assuring quality and longer shelf life of bread and bakery products. Publishing house of SUSU, Chelyabinsk, 160 pp. (in Russian).
Nechayev, A.P. 2015. Food chemistry: workbook. 6th edition. *Publishing House GIORD*, St. Petersburg. 672 pp. (in Russian).
Nilova, L.P. 2012.Functionality bakery products. Quality and safety. *Lema, St.Peterburg*, 179 pp. (in Russian).
Ostrikov, A.N. & Napolskikh, M.S. 2012. Differential thermal analysis for the study of bonds between moisture of vegetable-meat mixture conatining lupin, lentil and sublimated meat. *J. Problems of Contemporary Science and Practice. Vernadsky University* **4**(42), 335–339. (in Russian. English abstr.).
Palyvoda, S., Urchak, V., Golikova, T. & Fomenko, V. 2013. Study of the mechanism of the retarding effect on bread staling by using natural gums. *Food and Environment Safety* **4**(12), 300–310.
Rasmussen, P.H., Hansen, A. 2001. Staling of wheat bread stored in modified atmosphere. *Lebensmittel-Wissenschaft und-Technologie* **34**(7), 487–491.
Pashchenko, L.P. & Zharkova, I.M. 2006. Technology of bakery products. Publishing house: *KolosS.* Moscow, 389pp. (in Russian).
Schiraldi, A., Piazza, L. & Riva, M. 1996. Bread Staling: A Calorimetric Approach. *Cereal Chem.* **73**(1), 32–39.
Xie, F., Dowell, F.E. & Sun X.S. 2004. Using visible and near-infrared reflectance spectroscopy and differential scanning calorimetry to study starch, protein, and temperature effects on bread staling. *Cereal Chem.* **81**(2), 249–254.
Yurchak, V.G., Berzin, N. I. & Royter, I. M. 1988. The role of bound water in bread making and storage. Moscow, 20 pp. (in Russian).

*Agronomy Research* **15**(S2), 1399–1410, 2017

# An investigation into the effects of bioactive substances from vegetable oils on the antioxidant properties of bakery products

L. Nilova,* T. Pilipenko and S. Malyutenkova

Peter the Great St Petersburg Polytechnic University, Institute of Industrial Management, Economics and Trade, Graduate School of Commodity and Service, Novorossiyskaya Street 50, 194021 St Petersburg, Russia
* Correspondence: nilova_l_p@mai.ru

**Abstract.** This article discusses ways in which the antioxidant capacity of bakery products (otherwise referred to as 'BPs') can be increased by adding various types of vegetable oil to the dough: chosen as test oil was unrefined rice bran oil, unrefined pumpkin seed oil, and refined and deodorised sunflower oil. The authors conducted a study of fatty acid compositions and biologically active substances to be found in vegetable oils. The antioxidant properties of vegetable oils were analysed according to the following characteristics: the formation of the primary (peroxide value) and secondary (anisidine value) oxidation products; the oxidation coefficient (IR spectroscopy) which can be determined in the process of applying thermal treatment (with five hours of heating at 120 °C), which leads to the Vitamin E being destroyed. The biochemical composition of vegetable oils affected their resistance to the thermal oxidation process in the following sequence: unrefined rice bran oil > unrefined pumpkin seed oil > refined and deodorised sunflower oil. BPs were made from wheat flour dough with the addition of 4% of the corresponding vegetable oil and 5% of sugar, and were baked at two temperature regimes: at 200 °C and at 220 °C. The antioxidant activity of the BPs was determined by means of two methods: by chemiluminescence, and by DPPH radical assay. The antioxidant activity of the BPs varies depending on the vegetable oil being used, with the differences being revealed in the following way: BPs with unrefined pumpkin seed oil > BPs with unrefined rice bran oil > BPs with refined and deodorised sunflower oil. Any increase in the baking temperature reduced the antioxidant activity of the BPs; the antioxidant properties in the crust and the crumb were reduced at differing rates.

**Keywords:** sunflower oil, rice bran oil, pumpkin-seed oil, bioactive substances, bakery products, antioxidant properties

## INTRODUCTION

The search for natural antioxidants and their use in foodstuffs is becoming quite important in modern food technology research studies. Natural antioxidants, used as food additives, are safe for human health and can have a preventive medical effect when consumed regularly (Galkina, 2013; Nilova et al., 2016). The human habit of consuming bread and bakery products on a daily basis (Akhtar et al., 2011) makes the antioxidant properties of BPs an important object of study.

A good many research studies were devoted to the antioxidant properties of bread and bakery products. It was found that their antioxidant activity is determined by the

type of flour the particular BPs were made from, and by the flour extraction degree. Rye bread bakery products contain more antioxidants than wheat flour bakery products. The higher the degree of flour extraction, the higher the antioxidant properties of the bread (Horszwald et al., 2010; Murzahmetova et al., 2015). Bread and bakery products which are produced using wheat flour with a low degree of extraction contain the lowest volume of antioxidants (Dziki et al., 2014; Karrar, 2014). Various herbal additives are used to increase the BP antioxidant properties: pseudocereal flour (Chlopicka et al., 2012), powders, juices, extracts of berries (Meral & Doġan, 2012; Nilova et al., 2015), fruits (Belyavskaya & Rodicheva, 2013, Ummi et al., 2015), vegetables (Raba et al., 2007), seeds (Das et al., 2013; Jaisanthi et al., 2014), and other plants (Gawlik-Dziki et al., 2013). These ingredients display high antioxidant activity levels due to the water-soluble antioxidants – polyphenols and Vitamin C - but the content of these ingredients in bread and BPs is limited (Meral & Doġan, 2012; Dziki et al., 2014; Nilova et al., 2015) as they affect the sensory properties of the BPs. During the baking process, Vitamin C is almost completely destroyed, which leads to a decrease in the antioxidant properties.

It is possible to improve the antioxidant properties of BPs with the use of lipid-soluble antioxidants, which are present in vegetable oils. The use of oils and fats is essential in the production of BPs; fats carry out important technological and sensory functions (Pashchenko & Zharkova, 2006). Margarine, vegetable oils, shortenings, and other special oils and fats can be used as fatty components (Nechaev, 2013). Research on bakery products which are sold on the Italian market (Mignogna et al., 2015) showed that only in breads made with the use of vegetable oils were the required amount of tocopherols present. The use of margarine, even enriched with phytosterols, α-tocopherol and β-carotene, in BPs, increases the content of trans fatty acids up to 4.29 ± 1.48% – this exceeds the maximum allowed norms which have been adopted in the European Union (Quílez et al., 2006). Various refined deodorised oils are used in BP production in order to stabilise the sensory properties of BPs. (Nechaev, 2013; Mignogna et al., 2015). High temperature regimes used in refining and deodorising vegetable oils can decrease the levels of biologically active substances (BAS) (such as, for example, tocopherols) by as much as 30–70% (Zolochevsky, 2009). The baking process triggers the oxidation both of fatty acids and of BAS. The higher the quantity of BAS in vegetable oils, the less change occurs in them during the baking process (Caponio et al., 2013), and the lower is the level of destruction of tocoferols – the most active lipid-soluble antioxidants (Nyström et al., 2007; Nilova et al., 2013).

Rice bran or pumpkin seed oils can be used as the source of lipid-soluble antioxidants. Rice bran oil contains not only tocoferols and tocotrienols, but also γ-orysanol. The joint presence of γ-orysanol and tocopherols causes a synergistic effect in the antioxidant properties of rice bran oil, both when used in its natural form or as a food additive (Patel & Naik, 2004; Juliano et al., 2005; Lerma-Garcia et al., 2009). Pumpkin seed oil is a source of carotenoids, phospholipids, sterols, flavonoids, and tocopherols, but its chemical composition and antioxidant properties strongly depend on the raw products. Pumpkin seed oil obtained from *Cucurbita pepo* L contains the highest amounts of BAS – which increases its antioxidant properties (Rezig et al., 2012; Nawirska-Olszańska et al., 2013).

The aim of the present study is to investigate the composition of biologically active substances (BAS) and the antioxidant properties of rice bran oil and pumpkin seed oil,

and determines their role in enhancing the antioxidant properties of bakery products which are made using wheat flour.

## MATERIALS AND METHODS

### The materials and methods used in vegetable oil research

An unrefined rice bran oil and an unrefined pumpkin seed known as 'Dial-Export' oil, which are both produced by Butas LLC, were used as sources of lipid-soluble antioxidants.

The fatty acid composition of vegetable oil was determined by means of the gas chromatographic method, using the Agilent 6890 Series chromatograph (Agilent Technologies, Inc, USA), which was equipped with a DB-23 fused silica capillary column (60 m × 0.25 mm ID × 0.25 μm), and was used under the following conditions: the initial column temperature of 70 °C was gradually increased at a rate of 10 °C a minute$^{-1}$ until it reached a maximum of 180 °C. This temperature was held for ten minutes, and then increased at a rate of 5 °C a minute$^{-1}$ until it reached a final temperature of 220 °C which was held for eighteen minutes. Helium was used as the carrier gas at a flow rate of 4.6 ml per minute$^{-1}$.

The level of γ-orysanol was estimated by using the spectrophotometric method at a wavelength of maximum absorption of about 315 nm; the oil sample was previously dissolved in n-heptane (Srisaipet & Nuddagul, 2014); the levels of Vitamin E were estimated by means of the Emmeri-Engel spectrophotometric method with orthophenanthroline at a wavelength of maximum absorption of about 520 nm (Trineeva, 2013).

The qualitative composition of BAS was determined by means of the gas chromatography-mass-spectrometry method, using the MAESTRO 7820A gas chromatograph equipped with a 5975 mass selective detector with electron impact ionisation (70 eV), under the following conditions: a fused silica capillary column, Rxi – 5 ms (30 m × 0.25 mm × 0.25 μm); an injector temperature of 280 °C, a detector interface of 280 °C, an initial column temperature of 50 °C, a final column temperature of 280 °C, and a column heating rate of 15 °C min$^{-1}$. The carrier gas (Helium) was flown at a linear velocity of 1.0ml per minute$^{-1}$. The volume of the injected sample was 1 μl. The sample was injected with a split ratio of 1:30. Detection of the mass spectra was carried out on the full ion current in the positive ion scanning mode, in the range of masses 40–800 m z$^{-1}$. The mass spectra obtained from this were identified with the use of the equipment's electronic mass spectra library (library NIST11.L, DD2011.L).

The antioxidant properties of vegetable oils were determined by means of the detection of the primary (peroxide value) and secondary (anisidine value) oxidation products, and the thermal oxidation coefficient (IR-spectroscopy) in which Vitamin E is destroyed (after heating for five hours at 120 °C). The degree of thermal oxidation was recorded every hour.

The peroxide value (milliequivalents per kg) was determined by means of the potentiometric (BS EN ISO 27107:2010) method with the use of the ATP-02 automatic titrator, 'Akvilon', Russia. A test sample of vegetable oil was dissolved in isooctane and anhydrous acetic acid with the addition of potassium iodide.

The anisidine value was determined with the use of the SHIMADZU 1240 spectrophotometer (SHIMADZU, Japan) at a wavelength of 350 nm in cuvettes with an

optical path length of 10 mm in a test solution of oil in isooctane after its reaction with an acetate solution of paraanisidin (ISO 6885: 2016).

The oxidation coefficients were determined by calculating the ratio of value peaks in the IR spectra of plant oils: $K_1=A_{2850}/A_{3030}$; $K_2=A_{1465}/A_{3030}$; $K_3=A_{3450}/A_{2850}$; $K_4=A_{3450}/A_{1455}$ (Tokassado et al., 1979).

IR spectra were registered with the 'FSM 1202' FT-IR spectrometer (Monitoring LLC, Russia), with automatic calculation of the peaks in respect to the baseline. The spectra registration parameters were as follows: the spectral range was between 400–4,000 $cm^{-1}$; the number of scans carried out was twenty; the resolution was at $4cm^{-1}$; the mode was an interferogram. The absolute accuracy of the wave-number scale calibration did not exceed ± 0.1 $cm^{-1}$. Deviation of the 100% transmission line (1,950–2,050 $cm^{-1}$ at a resolution of 4 $cm^{-1}$, over the course of twenty scans) was less than % ± 0.5. The standard deviation of the 100% transmittance line (1,950–2,050 $cm^{-1}$ with a resolution of 4 $cm^{-1}$, over the course of twenty scans), did not exceed 0.025%. The resulting interferograms were converted to the absorption spectra and peaks were identified (Silverstein, 2011).

**The materials and methods used for research into bakery products**

Bakery products were made from wheat flour (gluten 28.9%, ash content 0.55), supplemented with 4% vegetable oil and 5% sugar. Test samples differed only by the type of vegetable oil additive. The following types of vegetable oils were used: refined and deodorised sunflower oil produced by EFKO FOODS PLC, Russia (the control); 'Dial-Export' rice bran refined oil, and 'Dial-Export' unrefined pumpkin seed oil (Butas LLC, Russia). Sample products of 100 g were baked under two temperature regimes: at 200 °C for 25 minutes, and at 220 °C for twenty minutes.

**A determination of water soluble antioxidants in bakery products**

The process of determining water soluble antioxidants in bakery products was conducted with the use of aqueous extracts of crumb and crust, which were obtained by extracting crumb and crust samples that had previously dried at 40 °C to a moisture content of 6.5–7.0%. A total of 250 mg crumb and crust samples were divided into a powder, diluted with 10ml of distilled water, and centrifuged for ten minutes at 3,500 rpm. A total of 1ml of the resulting extract was used for the purposes of antioxidant determination. The ratio of each subsequent dilution was 1:2.

The antioxidant activity of aqueous extracts was determined by means of the chemiluminescence method with the use of the BCL-06M biochemiluminometer (Nizhny Novgorod, Russia) in a model system which contained riboflavin, hydrogen peroxide, and ferrous iron (Putilina et al., 2006).

The chemiluminescent reaction of the riboflavin (that of the sample substrate) was initiated in the presence of ferrous and hydrogen peroxide ions. A total of 610μL of potassium phosphate buffer (with a pH of 7.5), 40μL of 10mM riboflavin solution, 100μL of physiological saline, and 25mM of ferric sulphate solution (II) (the model system) were added to the measuring cuvette of the BCL-06M biochemiluminometer. To initiate the process, a 0.1% $H_2O_2$ solution was used. In determining the antioxidant activity of the test samples, physiological saline was replaced with the relevant extracts at different concentration levels. The calibration curve was built up for trolox 97%, Acros Organics, USA.

A determination was carried out of free radical scavenging activity by means of the Glevindu method (Rogozhin & Rogozhina, 2015). This method is based on the principle of scavenging the DPPH radical (1,1-diphenyl-2-picrylhydrazyl).

Hydroalcoholic extracts of crumb and crust samples were prepared in the same manner as described above, but the extraction was carried out with the use of 50% ethanol solution. The DPPH solution was prepared by dissolving a 5mg test sample in 5ml of 16.4 M (96%) ethanol heated on a bain-marie until dissolved. A total of 0.2ml of the extract was added to 2 ml of DPPH solution. The resulting solution was incubated in the dark for thirty minutes, after which the absorbance levels of the resulting solution were measured at a wavelength of 517 nm using a SHIMADZU 1240 spectrophotometer (SHIMADZU, Japan). The results were calculated using the standard calibration curve for trolox (trolox 97%, Acros Organics, USA).

Each bakery product sample has undergone researched in triplicate. The accuracy of the experimental data was evaluated by using mathematical statistical methods in Microsoft Excel. The data gained through this process are presented with a confidence coefficient of 0.95.

## RESULTS AND DISCUSSION

All of the vegetable oil samples belonged to the linoleic-oleic group of vegetable oils by fatty acid composition (Table 1). Similar fatty acids – linoleic, oleic, and palmitic – dominated amongst these. The levels of other fatty acids did not exceed 5%. The pumpkin seed and sunflower oils were richer in linoleic acid, while the rice bran oil showed more oleic acid. The preponderance of linoleic acid with its two double bonds, especially in sunflower oil, makes it less resistant to thermal treatment. The oxidation depends on the level of biologically active substances with antioxidant properties.

**Table 1.** Fatty acid composition of vegetable oils*, %

| Type of oil | Fatty acid | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $C_{14:0}$ | $C_{16:0}$ | $C_{16:1}$ | $C_{18:0}$ | $C_{18:1}$ | $C_{18:2}$ | $C_{18:3}$ | $C_{20:0}$ | $C_{20:1}$ | $C_{20:2}$ |
| Rice bran | 0.89 | 17.35 | 0.19 | 2.05 | 43.71 | 33.10 | 1.01 | 0.60 | 0.53 | 0.57 |
| Pumpkin-seed | - | 10.47 | - | 3.87 | 32.95 | 52.34 | - | 0.37 | - | - |
| Sunflower | 0.07 | 6.33 | 0.08 | 4.51 | 18.49 | 69.28 | 0.09 | 0.28 | 0.14 | 0.73 |

* The difference between the values of two determi-nations for constituents present in excess of 5% (m $m^{-1}$) not exceed 1.5% (relative) of the determined value; for constituents present in smaller quantities, the difference not exceed of 0.05% (m $m^{-1}$) of the determined value

Natural vegetable oils contain bioactive substances (BAS) such as tocopherols and tocotrienols, sterols, phospholipids, and carotenoids which display antioxidant properties. However, during the production process, especially with the use of certain refining methods, antioxidants in vegetable oils can sufficiently deteriorate (Zolochevsky, 2009). The oils being studied differed by their BAS quantitative and qualitative composition (Table 2). Refined and deodorised sunflower oil hardly contained any antioxidants, except Vitamin E, (α-tocopherol only), the amount of which was less 1.79 and 3.37 times respectively when compared to rice bran and pumpkin seed oil.

Although it contained 1.88 times less Vitamin E than pumpkin seed oil, rice bran oil is richer in antioxidant properties due to the presence of γ-orysanol and sterols. Pumpkin seed oil has the highest content of Vitamin E, represented primarily by β- and γ-tocopherols and squalene. Of phytosterols, only β- and γ-sitosterol were identified. Vegetable oil BAS do not only help to prevent oxidative processes during baking, but also enrich bakery products with antioxidants. The antioxidant properties of vegetable oils were studied by the intensity of the oxidative processes during thermal exposure at 120 °C. At this temperature, Vitamin E begins to decay. Fig. 1 shows the data taken from studies of various oil peroxide and anisidine values registered during the thermal oxidation process.

During the first hour of thermal oxidation almost no changes in peroxide values occurred in all test vegetable oil samples.

**Table 2.** Biologically active substances of vegetable oil samples

| Bioactive substances | Type of oil | | |
|---|---|---|---|
| | Rice bran | Pumpkin-seed | Sunflower |
| Vitamin E, mg $100g^{-1}$ | 98.8 ± 1.8 | 186.2 ± 2.9 | 55.3 ± 1.3 |
| γ-orysanol, mg $100g^{-1}$ | 565.49 ± 9.12 | - | - |
| Qualitative composition | | | |
| Tocopherols*:* | | | |
| α-tocopherol | + | - | + |
| β-tocopherol | - | + | - |
| γ- tocopherol | + | + | - |
| Sterols*:* | | | |
| β-sterol | + | + | - |
| γ-sterol | + | + | - |
| Campesterol | + | - | - |
| Stigmasterol | + | - | - |
| Squalene | - | + | - |

During the next few hours, the intensive formation of peroxides occurred only in sunflower oil due to the high content of linoleic acid in which the oxidation rate is 27 times higher than in oleic acid (Nechaev, 2013). The only antioxidant present in the sunflower oil – α-tocopherol – is oxidised at this temperature at a faster rate than any other tocopherol. Its quantity is sufficient to inhibit the oxidation processes for one hour only, so after two hours of thermal oxidation the peroxide values increased by almost double. During the third hour the oxidation rate increased sharply, and the peroxide value rose beyond acceptable limits. Further changes were associated with a higher rate of peroxide destruction when compared to their formation, leading to an increase of secondary oxidation products. During the fourth hour of thermal oxidation in the sunflower oil the anisidine value exceeded three units.

In rice bran and pumpkin seed oil the oxidation rate was significantly lower due to the presence of a wide range of BAS which was rich in antioxidant properties. Changes in the values of peroxide figures were insignificant and did not exceed three milliequivalents per kg during all five hours of thermal oxidation. The oxidation processes in pumpkin seed oil were more pronounced when compared to rice bran oil,

especially in terms of the formation of secondary oxidation products. During the fourth hour their number increased dramatically.

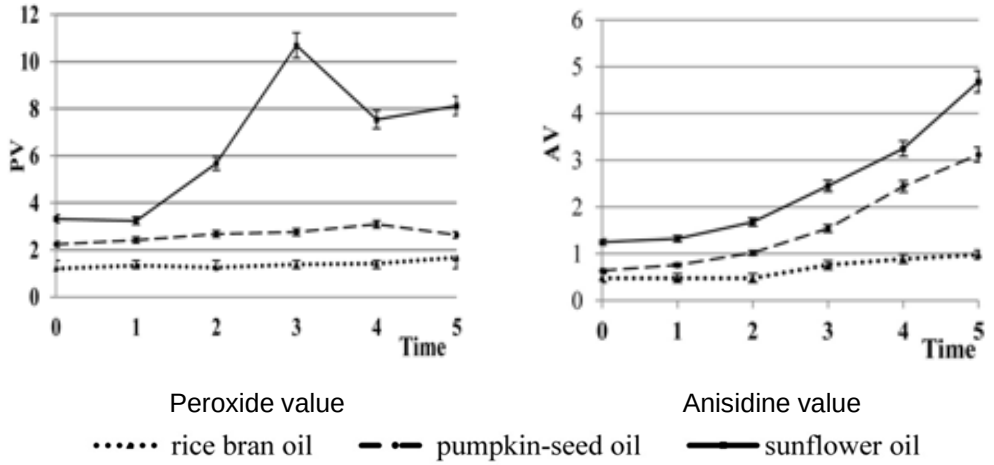



**Figure 1.** Peroxide (PV) and anisidine (AV) values during thermal oxidation process (hour).

The different character of the oxidative processes in the test vegetable oil samples was reflected in the change of IR absorption spectra during the five hour thermal oxidation process (Fig. 2).

Visible changes in sunflower oil occurred after two hours of thermal oxidation; in the pumpkin seed oil this took place in the fourth hour; and in the rice oil it happened only by the end of the fifth hour. In order to be able to compare the degree of oxidation in vegetable oils which were different in their original composition of fatty acids and bioactive substances, Tokassado et al. (1979) introduced oxidation coefficients which reflect the intensity of the absorption bands (in the IR spectrum) of the more oxidation-susceptible components versus the more stable ones (C-H deformation and stretching vibrations in the $CH_2$ group, at frequencies of 1,465 and 2,850 $cm^{-1}$).

Oxidation coefficients for the 3,008 $cm^{-1}$ band ($K_1$ and $K_2$) mark the oxidative chain initiation stage, while for the 3,450 $cm^{-1}$ band ($K_3$ and $K_4$), they mark the oxidative chain termination stage, which results in the formation of the secondary products of oxidation. Oxidation coefficients in the vegetable oil samples were calculated at the start of visible changes in the IR spectra (Table 3). Subsequent oxidation could have led to the disappearance of stretching vibrations in the 3,450 $cm^{-1}$ band. Therefore, the pumpkin seed oil sample, in the fifth hour of thermal oxidation, demonstrated a lack of this band, probably due to polymerisation processes.

The depth and velocity of oxidation are directly dependent upon the amount of polyunsaturated fatty acids and the degree of their unsaturation. Therefore, the oxidation of linoleic acid, which is prevalent in sunflower oil, is more intense due to the opening of double bonds and the formation of oxidised molecular products that occur earlier than they do in oleic acid. This resulted in an increase of oxidation coefficient values in sunflower oil after two hours of thermal oxidation. For the 3,007 $cm^{-1}$ band, the oxidation coefficients ($K_1$ and $K_2$) in sunflower oil after two hours of heating were greater than

they were in pumpkin seed oil after four hours of heating, or in rice bran oil after five hours of thermal oxidation.

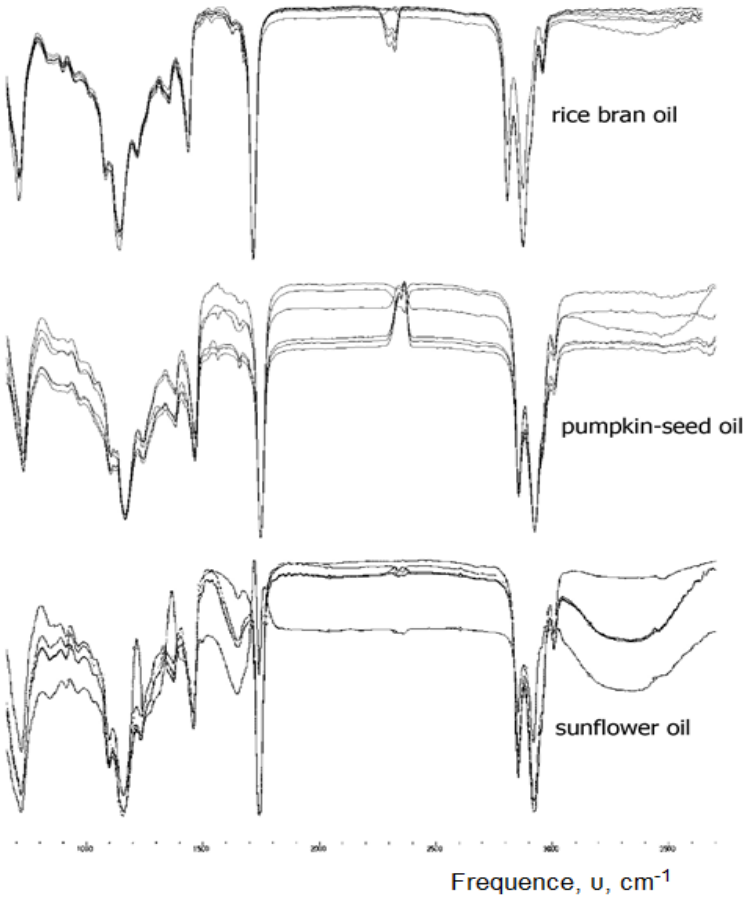



**Figure 2.** IR spectra of vegetable oils for 5 hours thermal oxidation.

The absorption area of 3,200–3,700 $cm^{-1}$ is characteristic for stretching vibrations in the free OH group. The appearance of marked fluctuations in this area can be attributed to the formation of the secondary products of lipid oxidation such as, for example, hydroperoxides and hydroxy compounds (Silverstein, 2011). But stretching vibrations in this area were different for different oils. In sunflower and pumpkin seed oil, the indexes $K_3$ and $K_4$ reached almost similar values, but within different time periods – after two and four hours respectively. In rice bran oil changes occurred only after five hours of thermal oxidation, with a demonstrated velocity a hundred times less than for the 3,008 $cm^{-1}$ band ($K_1$ and $K_2$). This shows that the formation of secondary oxidation products ($K_3$ and $K_4$) in the oil being studied occurred a hundred times less intensively than the process of oxidative chain emergence ($K_1$ and $K_2$).

**Table 3.** Oxidation coefficients of the vegetable oil samples at the start of visible changes in the IR spectra

| Type of oil | Oxidation time, h | Oxidation coefficients | | | |
|---|---|---|---|---|---|
| | | 3,007 $cm^{-1}$ band | | 3,450 $cm^{-1}$ band | |
| | | **$K_1$** | **$K_2$** | **$K_3$** | **$K_4$** |
| Rice bran | 5 | 4.174 ± 0.090 | 3.275 ± 0.080 | 0.014 ± 0.001 | 0.023 ± 0.001 |
| Pumpkin-seed | 4 | 3.223 ± 0.078 | 2.202 ± 0.049 | 2.299 ± 0.030 | 3.469 ± 0.051 |
| Sunflower | 2 | 4.458 ± 0.105 | 3.812 ± 0.076 | 2.364 ± 0.044 | 3.541 ± 0.068 |

Thanks to their resistance to thermal treatment, the test vegetable oil samples were categorised in the following order: unrefined rice bran oil > unrefined pumpkin seed oil > refined and deodorised sunflower oil.

Biologically active substances which are present in vegetable oils affected the antioxidant properties of BPs (Table 4). The unrefined rice bran oil and pumpkin seed oil increased antioxidant properties in bakery products by almost double (by means of the DPPH method) when compared to bakery products that contained sunflower oil. By means of the BAS effect on the antioxidant activity of bakery products, the following sequence was noted: BPs with pumpkin oil > BPs with rice oil > BPs with sunflower oil.

**Table 4.** Antioxidant activity of bakery products, μg Trolox per g DM

| Bakery products | DPPH-radical assay | | Chemiluminescence | |
|---|---|---|---|---|
| | crust | crumb | crust | crumb |
| Baking at 220 °C: | | | | |
| Rice bran oil | 7.12 ± 0.12 | 8.06 ± 0.15 | 10.98 ± 0.21 | 11.77 ± 0.25 |
| Pumpkin-seed oil | 7.92 ± 0.11 | 9.84 ± 0.15 | 23.00 ± 0.24 | 16.14 ± 0.23 |
| Sunflower oil | 3.18 ± 0.07 | 4.56 ± 0.10 | 7.13 ± 0.17 | 10.51 ± 0.22 |
| Baking at 200 °C: | | | | |
| Rice bran oil | 7.61 ± 0.16 | 8.49 ± 0.14 | 11.76 ± 0.19 | 12.40 ± 0.21 |
| Pumpkin-seed oil | 8.52 ± 0.15 | 10.36 ± 0.13 | 21.82 ± 0.22 | 17.01 ± 0.28 |
| Sunflower oil | 3.44 ± 0.07 | 5.11 ± 0.08 | 7.75 ± 0.17 | 11.13 ± 0.26 |

Due to the high temperature impact, the antioxidant activity in the crust was less than that of the crumb. During baking the crumb is warmed up to only 95 °C while the crust gets heated to 220 °C (Pashchenko & Zharkova, 2006). Vitamin E, contained in the crust, is destroyed more intensively at such high temperatures. Therefore the antioxidant activity of the crust which contained pumpkin seed oil was less than it was in the crumb, by a factor of 19.51%, while the crust with its rice bran oil which contained less Vitamin E, displayed a difference of only 11.66%. But despite the synergism of γ-orysanol and Vitamin E in rice bran oil (Juliano et al, 2005), the antioxidant activity of BPs which contained rice oil was lower than it was with BPs which contained pumpkin oil. Apparently, much of the BAS antioxidant activity was spent to prevent the oil's fatty acids from being oxidised.

The baking of BPs at a lower temperature (200 °C) led to an increase in the antioxidant activity values, both in the crumb and in the crust. During baking at a higher temperature, the crumb displayed better antioxidant activity than did the crust.

The antioxidant activity of aqueous crust and crumb extracts depends, firstly, on the amount of phenolic compounds in the flour (Raba et al., 2007; Horszwald et al., 2010) and, secondly, on melanoidins which are formed during baking (Belyavskaya et al., 2013; Nilova et al., 2015). Wheat flour, especially of a low extraction degree, contains the least amount of phenolics (Dziki et al., 2014). In bakery products which are made from the same flour, the antioxidant activity values of aqueous extracts with different vegetable oils depend on the amount of melanoidins formed during baking. The antioxidant activity of the crumb aqueous extracts, detected by means of the chemiluminescent method, was higher than in the crust, in BPs with rice bran and sunflower oil by 7.19 and 4.74% respectively. Contrary to this finding, in BPs with pumpkin seed oil the crust displayed a higher antioxidant activity than did the crumb. A lower baking temperature increased the antioxidant activity of aqueous extracts, more so in the crumb than in the crust.

## CONCLUSIONS

Unrefined vegetable oils – rice bran oil and pumpkin seed oil – contain a wide range of biologically active substances which are rich in antioxidant properties. They differ both in terms of the quantitative content of Vitamin E, which is 1.88 times higher in the pumpkin seed oil than it is in the rice bran oil, and by their qualitative composition. Pumpkin seed oil contains β- and γ-tocopherols, while rice bran oil is rich in α- and γ-tocopherols. The rice oil contains γ-orysanol and sitosterol, while the pumpkin seed oil shows the presence of squalene and only β- and γ-sitosterol from the sterols group.

The lack of biologically active substances (with the exception of α-tocopherol), and the high content of linoleic acid in the refined and deodorised sunflower oil, led to accelerated oxidative processes during thermal treatment. The visible changes in the IR spectra occurred after two hours of thermal oxidation, and by the third hour the peroxide value exceeded ten milliequivalents per kg.

A wide range of biologically active substances in the rice bran oil and pumpkin seed oil slows down oxidative processes that occur during thermal oxidation. In the rice bran oil the oxidative processes proceeded at a slower rate, leading to changes in the IR spectra only after five hours of thermal oxidation. During thermal treatment, processes prevailed which were characteristic of the initiation stage of the oxidative chain reaction rather than of the termination stage: oxidation coefficients for the 3,008 $cm^{-1}$ band ($K_1$ and $K_2$) exceeded those for the 3,450 $cm^{-1}$ band ($K_3$ and $K_4$) by double the amount. In pumpkin seed oil, changes in the IR spectra occurred after four hours of thermal treatment: processes which were characteristic of the initiation stage of oxidative chain reaction and the termination stage proceeded at a similar rate. The oxidation values differed only slightly, and were of the same order.

Unrefined rice bran oil, pumpkin seed oil, and sunflower oil which is used as additives in bakery products contributed to an increase in antioxidant activity in BPs in the following order: BPs with pumpkin seed oil > BPs with rice bran oil > BPs with sunflower oil. The total antioxidant activity (as determined by means of the DPPH method) was higher in the crumb than it was in the crust, and increased with a decreasing baking temperature. The same tendency was characteristic of the antioxidant activity in aqueous extracts of crust and crumb as determined by chemiluminescence. The only

exceptions were BPs with pumpkin seed oil: the antioxidant activity of aqueous extracts in the crust was higher than that in the crumb.

## REFERENCES


Akhtar, S., Anjum, F.M. & Anjum, M.A.A. 2011. Micronutrient fortification of wheat flour: recent development and strategies. *Food Research International* **44**, 652–659.

Belyavskaya, I.G. & Rodicheva, N.V. 2013. Determination of antioxidant capacity of bakery products vegetables processing. *Khleboproducty* **11**, 52–53 (in Russian, English abstr.).

Caponio, F., Giarnetti, M., Summo, C., Paradiso, V.M., Cosmai, L. & Gomes, T. 2013. A comparative study on oxidative and hydrolytic stability of monovarietal extra virgin olive oil in bakery products. *Food Research International* **54**(2), 1995–2000.

Chlopicka, J., Pasko, P., Gorinstei, Sh., Jedryas, A. & Zadrodzki, P. 2012. Total phenolic and total flavonoid content, antioxidant activity and sensory evaluation of pseudocereal breads. *LWT – Food Science and Technology* **46**, 548–555.

Das, L., Raychaudhuri, U. & Chakraborty, R. 2013. Herbal Fortification of Bread with Fennel Seeds**.** *Food Technol. Biotechnol.* **51**(3), 434–440.

Dziki, D., Różyło, R., Gawlik-Dziki, U. & Świeca, M. 2014. Current trends in the enhancement of antioxidant activity of wheat bread by the addition of plant materials rich in phenolic compounds. *Trends in Food Science & Technology* **40**(1), 48–61.

Galkina, O.V. 2013. The specific features of free-radical processes and the antioxidant defense in the adult brain. *Neurochemical Journal* **7**(2), 89–97.

Gawlik-Dziki, U., Swieca, M., Dziki, D., Baraniak, B., Tomiło, J. & Czyz, J. 2013. Quality and antioxidant properties of breads enriched with dry onion (*Allium cepa L.*) skin. *Food Chemistry* **138**, 1621–1628.

Horszwald, A., Morale, F.J., Castillo, M.D. & Zielinski, H. 2010. Evaluation of antioxidant capacity and formation of processing contaminants during rye bread making. *Journal of Food and Nutrition Research* **49**(3), 149–159.

Jaisanthi, J. & Banu, T.A. 2014. Phytonutrient Composition, Antioxidant Activity and Acceptability of Baked Product Incorporated with Grape Seed Extract. *Journal of Human Nutrition & Food Science* **2**(6), 1049–1054.

Juliano, C., Cossu, M., Alamanni, M.C. & Piu, L. 2005. Antioxidant activity of gamma-oryzanol: mechanism of action and its effect on oxidative stability of pharmaceutical oils. *International journal of Pharmaceutics* **299**(1), 146–154.

Karrar, E. M. 2014. A Review on: Antioxidant and Its Impact during the Bread Making Process. *International Journal of Nutrition and Food Sciences* **3**(6), 592–596.

Lerma-Garcia, M.J., Simo-Alfonso, J.M., Mendonsa, E.F. & Ramis-Ramos, C.R.B. 2009. Composition, industrial processing and applications of rice bran γ-oryzanol. *Food Chemistry* **115**, 389–404.

Meral, R. & Doğan, İ.S. 2012. Karadut (*Morus nigra*) Katkılı Ekmeğin Antioksidan Aktivitesi ve Fenolik Kompozisyonu**.** *Iğdır Univ. J. Inst. Sci. & Tech.* **2**(4), 43–48 (in Turkish, English abstract).

Mignogna, R., Fratianni, A., Niro, S. & Panfili, G. 2015. Tocopherol and tocotrienol analysis as a tool to discriminate different fat ingredients in bakery products. *Food Control* **54**, 31–38.

Murzahmetova, M.K., Tayeva, A.M., Baimaganbetova, G.B., Nabiyeva, Z.S., Kizatova, M.Z., Kulazhanov, K.S. & Vitavskaya, A.V. 2015. Antioxidant Activity of Breads. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* **6**(3), 1020–1025.

Nawirska-Olszańska, A., Kita, A., Biesiada, A., Sokół-Łętowska, A. & Kucharska, A.Z. 2013. Characteristics of antioxidant activity and composition of pumpkin seed oils in 12 cultivars. *Food Chemistry* **139**(1), 155–161.

Nechaev, A.P. 2013. Food ingredients in production of bakery products. *Deli plus*, Moscow. 527 pp.

Nilova, L., Orlova, O. & Nasonova, J. 2015. The role cyclic amides in the formation of antioxidant capacity of bakery products. *Agronomy Research* **13**(4), 1020–1030.

Nilova, L.P., Pilipenko, T.V., Markova, K.Y. & Sikoev, Z.H. 2013. Functional and Technological Properties of a New Generation Oils. *Fat and oil processing industri* **6**, 22–27 (in Russian, English abstract).

Nilova, L.P. & Pilipenko, T.V. 2016. Evaluation antioxidant properties enriched bakery products in experiment on laboratory animals. *Problems of Nutrition* **85**(6), 39–47 (in Russian, English abstract).

Nyström, L., Achrenius, T., Lampi, A.M., Moreau, R.A. & Piironen, V. 2007. A comparison of the antioxidant properties of steryl ferulates with tocoferol at high temperatures. *Food Chemistry* **101**, 947–954.

Patel, M. & Naik, S.L. 2004. Gamma-oryzanol from rice bran oil. *Journal of Scientific and Industrial research* **63**, 569–578.

Pashchenko, L.P. & Zharkova, I.M. 2006. Technology of bakery products. *Publishing house 'Colossus', Moscow.* 389pp. (in Russian).

Putilina, P.E., Galkina, O/V., Eschenko, I.D., Digi, G.P., Krasovskaya, I.E. & Prokopenko, V.M. 2006. Workshop on free radical oxidation. *St.Peterburg state University*, St.Peterburg. 82–83 (in Russian).

Quílez, J., Ruiz, J.A., Brufau, G. & Rafecas, M. 2006. Bakery products enriched with phytosterols, α-tocopherol and β-carotene. Sensory evaluation and chemical comparison with market products. *Food Chemistry* **94**(3), 399–405.

Raba, D. N., Moigrădean, D., Poiană, M.-A., Popa, M. & Jianu, I. 2007. Antioxidant capacity and polyphenols content for garlic and basil flavored bread. *Journal of Agroalimentary Processes and Technologies* **13**(1), 163–168.

Rezig, L., Chouaibi, M., Msaada, K. & Hamdi, S. 2012. Chemical composition and profile characterization of pumpkin (*Cucurbita maxima*) seed oil. *Industrial Crops and Products* **37(**1), 82–87.

Rogozhin, V.V. & Rogozhina, T.V. 2015. Workshop on biochemistry of agricultural production. *GIORD.* St. Petersburg 480 pp. (in Russian).

Silverstein, P. 2011. Spectrophotometric identification of organic compounds. *Binom. Laboratoriya znaniy*, Moscow. 557 pp. (in Russian, transl. from English).

Srisaipet, A. & Nuddagul, M. 2014. Influence of Temperature on Gamma-Oryzanol Stability of Edible Rice Bran Oil during Heating. *International Journal of Chemical Engineering and Applications* **5**(4), 303–306.

Tokassado, M., Horikawa, K. & Masayma, S. 1979. A new spectral measessing oxidative stability of oil fat. *J. Jap. Oil Chem*. **4**(28), 291–299.

Trineeva, O.V. 2013. Methods of vitamin E analysis. *Scientific journal Proceedings of Voronezh State Univercity. Series: Chemistry. Biology. Pharmacy*. **1**, 212–224 (in Russian).

Ummi, K.I., Ruzitah, M. S., Suzihaque, M.U.H. & Syafiza, A.H. 2015. Effect of Radiation Heat on the Chemical and Physical Properties of Bread Enhanced with Garcinia Mangostana Pericarp Powder. *Procedia - Social and Behavioral Sciences* **195**, 2652–2659.

Zolochevsky, V.G. 2009. Change of structure and properties of oils at deodorization in schemes of physical refining. *Oils and fats* **10**, 8–11 (in Russian).

*Agronomy Research* **15**(S2), 1411–1419, 2017

# The influence of k-casein genotype on the coagulation properties of milk collected from the local Latvian cow breeds

S. Petrovska[1,*], D. Jonkus[1], J. Zagorska[2] and I. Ciprovica[2]

[1]Latvia University of Agriculture, Faculty of Agriculture, Institute of Agrobiotechnology, Street Liela 2, LV-3001 Jelgava, Latvia
[2]Latvia University of Agriculture, Faculty of Food Technology, Department of Food Technology, Street Rigas 22, LV-3004 Jelgava, Latvia
*Correspondence: solvitapetrovska@gmail.com

**Abstract.** Cheese production is becoming increasingly more diversified all over the world. The information on milk coagulation properties among Latvian dairy cow breeds and its suitability for cheese production still remains unclear. At the same time, milk with good renneting properties collected from the native Latvian cows can be used for the production of Protected Denomination of Origin (PDO) cheeses. The purpose of this research was to analyse the influence of the milk protein genotypes present in Latvian native cattle breeds on the milk coagulation properties. The Data was collected in 2016 from 56 Latvian brown, 26 Latvian blue and 13 Holstein black and white cows i. Highest frequency of AA κ-casein genotype was found in Latvian brown breed (0.593), while AB genotype was more often present in the Latvian blue breed (0.636). It has been found that the presence of κ-casein genotype resulted in no significant difference in milk composition and milk coagulation properties among studied cattle breeds. We have observed a tendency that the most desirable milk coagulation properties were present in BB genotype. A significant effect of breed on milk composition has been found ($p < 0.05$). Milk yield of Holstein Black and White was 32.0 ± 2.99 kg, while in Latvian blue it was only 17.6 ± 1.32 kg. Higher milk yield was obtained in Latvian blue breed in comparison to that of the brown breed – 19.10 ± 0.76 kg. Better milk coagulation properties were observed in Latvian brown breed – shorter milk renneting time (16.86 ± 1.15 min), highest curd yield (24.0 ± 0.79%) and curd firmness (3.21 ± 0.17 N).

**Key words:** Latvian brown cows, Latvian blue cows, milk coagulation properties

## INTRODUCTION

The production of cheese has grown in recent years and cheese plays an important role in the economics of Latvian dairy industry. The technological properties of individual milk samples for cheese making have traditionally been analysed through the assessment of milk coagulation properties.

Milk coagulation properties are influenced by various factors, whilst the animal breed remains the most important factor (Malchiodi et al., 2014). The B allele of κ-casein is associated with more desirable coagulation properties and higher protein content in milk (Azevedo et al., 2008). A allele increases milk yield and decreases protein content in it. A and B alleles are prevalent compared to E allele. Negative effect of E allele on milk coagulation properties (more non-coagulated milk samples, longer milk renneting

time and lower curd yield) were found in previous studies (Davoli et al., 1990; Macheboeuf et al., 1993; Ikonen et al., 1999).

The frequencies of genotypes and alleles of κ-casein vary across the breeds AA genotype and A allele are occurring in high-productivity dairy breeds (Tsiaras et al., 2005; Bonvillani et al., 2010). Choobini et al., (2014) have found that AA genotype frequency was 0.174 in Holstein, while AB and BB genotypes frequencies were 0.304 and 0.261, respectively. Genotypes with E allele were with low frequencies, AE and EE genotypes were found with frequencies 0.043 and 0.087 (Choobini et al., 2014). Frequencies of A allele were 0.790 and 0.695 in Estonian Holstein and Estonian Native, while frequencies of B allele were 0.138 and 0.305, respectively (Jõudu et al., 2007).

A preliminary comparison of milk composition among Latvian dairy breeds in earlier studies (Cielava et al., 2015) shows that the milk collected from the Latvian native cows has a number of advantages, despite the fact that the limited numbers of studies are available on the suitability of Latvian native cow's milk. The purpose of this research was to analyse the influence of κ-casein polymorphism present in Latvian native cattle and Holstein Black and White breeds on the milk coagulation properties.

## MATERIALS AND METHODS

Individual milk samples ($n$ = 95, morning milking) from the Latvian cow breeds (56 Latvian brown (LB), 26 Latvian blue (LZ) and 13 Holstein black and white cows (HBW) were collected across different Latvian regions during the summer of 2016 July to August, 2016) period.

Cows were kept in small and extensive farms – on average 7 animals in each farm. During the summer cows were grazed in cultivated pastures, fed with grain meals and minerals. LB and LZ cows according bloodiness were animal genetic resources in Latvia. LB cows were with ≥ 50% LB bloodiness and the rest were Danish red, Angeln bloodiness. LZ cows were with ≥ 50% LZ bloodiness and rest was Lithuanian grey, Tyrol, Latvian brown. HBW cows were with Holstein bloodiness 75 to 90% and rest was Latvian brown, Holstein Red and White bloodiness. Cows with AA genotype were 134 ± 12.6 days in milk, with AB genotype 130 ± 13.5 days in milk, but cows with BB genotype 139 ± 14.1 days in milk. Milking was done two times per day automatically. Cows with AA genotype were 2.9 ± 0.25 parity, cows with AB genotype 2.7 ± 0.34 parity, while cows with BB genotype 3.1 ± 0.56 parity. Live weight of LB, LZ and HBW was 557 ± 10.6 kg, 520 ± 12.4 kg and 620 ± 6.5 kg, respectively.

Approximately 120 LB and 100 LZ cows were included in animal genetic resources savings programme in Latvia. Milk samples were taken from a number of those cows and analysed to determine the coagulation properties of milk produced by the native Latvian cows.

Blood samples for *CSN3* genotyping were analysed in Scientific Laboratory of Molecular Biology and Microbiology of the Latvian University of Agriculture (LUA). Blood samples were collected from jugular vein (*Vena Jugularis*) in 10 mL sterile vacuumtainers. Genotyping was done using Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) and electrophoresis on 3% agarose gel. The identification of *CSN3* single nucleotide polymorphisms (SNPs) was done according to Velmala et al. (1993). SNPs at positions 13104 and 13124 were examined to determine the nucleotide changes (A->C and A->G), which determine *CSN3* alleles A, B and also

E. For digestions were used endonuclease *HinfI* to detect the presence of alleles A and B, and endonuclease *BsuRI* to detect the presence of the allele E.

Protein and fat content, curd yield and curd firmness were detected in Faculty of Food Technology of LUA. Protein content was detected according to ISO 8968-1:2014 using Kjeltec$^{TM}$ 2100 (Foss, Denmark). Fat content was detected according to ISO 488:2008 using centrifuge (Funke Gerber, Germany).

Curd yield (%) was calculated by weight of curd obtained from milk.

The rennet (CHY-MAX 1000 IMCU/ml, Chr. Hansen, Denmark) used in the analyses of curd firmness was diluted 1:100 (v/v) and added 0.2 ml to 10 ml of milk. The curd firmness (in Newtons) was determined after 30 min of milk renneting at 35 °C using Texture Analyser TA-HD plus (Stable Micro System, UK). Compression method for determination of curd firmness (technical data – disc A/BE – d45, test speed 1.0 mm $s^{-1}$, distance in the depth of curd sample 8 mm) was used.

Renneting time in minutes was analysed using 1:100 (v/v) of rennet solution into water and measuring the time until flocculation of milk was started at 35 °C. For interpretation of results, all samples were divided into four groups (fast, average, slow and non-coagulating milks) based on the time devoted for the clotting of samples. The assessment of clotting time was as follows: fast = flocculation formation during 10 min, average = 15 min, slow = more than 15 min, and non-coagulating = samples that did not coagulate at all.

Statistical data processing was carried out using MS Excel and SPSS 15.0 for Windows. The differences between group were significant if $p < 0.05$.

## RESULTS AND DISCUSSION

Allelic frequencies of A, B, E alleles were 0.778, 0.213 and 0.009 in LB breed. The frequency of B allele was higher in LZ breed – 0.364, respectively. Allele E was not found in LZ and HBW breed. A low frequency of E allele presence was observed in Latvian cow breeds. Researchers from different countries also confirms low frequencies of E allele (Lien et al., 1999; Zepeda-Batista et al., 2014)

The frequency of A allele increases in high-productivity animal selection. Genome selection is the instrument of increasing the frequency of B allele – focusing on dairy breeds that produce milk suitable for cheese production (Kübarsepp et al., 2005). Frequency of B allele in Lithuanian dairy cows was 0.23 higher comparing to that of Latvian breeds (Morkūnienė et al., 2016). Smiltina and Grislis (2010) have observed the frequency of AA genotype equal to 0.467 in LB and 0.465 in LZ breed, while frequencies of AB genotype were 0.433 in LB and 0.0437 in LZ.

Genotype frequency of AA was higher in LB breed compared with LZ breed – 0.593 and 0.318, respectively. Frequency of genotype BB was highest in LZ breed. Frequency of AA genotype was 0.615 in HBW, while frequency of AB was 0.385 (Table 1). According to previous research (Tsiaras et al., 2005), AA genotype is widespread in Holstein breed. Frequency of AA genotype was observed 0.89, while frequency of AB was only 0.11 in Holstein.

We did not analyse the influence of AE κ-casein genotype on milk productivity owing to low number of cows with this genotype in our sample.

Milk coagulation properties determine the quality of cheese produced from that milk yield. We have found a significant influence ($p < 0.05$) of κ-casein genotype on

milk productivity, milk quality and milk coagulation properties. We have found higer milk yield in AA genotype, while lower milk yield was in BB genotype group – 19.3 ± 0.86 and 17.1 ± 3.61 kg, respectively. Notably, Lithuanian researchers have similarly found that κ-casein genotype significantly influences the milk yield from dairy cows. Highest milk yield from cows with AA genotype, compared with AB and BB κ-casein genotypes, was found in Lithuanian dairy cows (Pečiulaitienė et al., 2007).

**Table 1.** κ-casein alleles and genotypes frequencies in analysed dairy cows

| Alleles and genotypes | Frequency of alleles and genotypes | | |
|---|---|---|---|
| | Latvian brown ($n$ = 56) | Latvian blue ($n$ = 26) | Holstein Black and White ($n$ = 13) |
| A | 0.778 | 0.636 | 0.808 |
| B | 0.213 | 0.364 | 0.192 |
| E | 0.009 | – | – |
| AA | 0.593 | 0.318 | 0.615 |
| AB | 0.352 | 0.636 | 0.385 |
| BB | 0.037 | 0.045 | – |
| AE | 0.019 | – | – |

Similar results were obtained in Sitkowska and co-authors (2013) study on κ-casein genotype influences on milk protein content. Highest protein content was observed in BB and AB genotype group – 3.47 ± 0.24% and 3.46 ± 0.83%, respectively.

Shorter milk renneting time was observed in BB genotype group 13.31 ± 1.84 min, whilst the longest time was observed in AA genotype group ($p < 0.05$, Table 2)

In the current research all milk samples were distributed in the following manner: fast flocculation was 15%, average – 30%, slow – 47%.

We observed 8% non-coagulated milk samples, mostly from cows with AA genotype (two samples were from AB genotype and six from AA genotype). Three non-coagulated samples were from HBW and LZ breed, two from LB. Similar results were found in previous researches, Ikonen et al. (2004) and Cassandro et al. (2008) reported that 7.5% to 13.2% Holstein cow's milk samples are not coagulated. Kübarsepp et al. (2006) has found that 4.42% non-coagulated milk came from cows with AE κ-casein genotype and 4.04% from AA genotype cows. Milk from cows with BE and BB genotype was not coagulated (only 1.22% and 1.36%). Zannoni and Annibaldi (1981) recommended that optimal value of milk coagulation time is 13 min and whilst we have obtained similar results only in the BB genotype group – 13.31 ± 1.84 min.

Curd yield is a very important parameter for cheese production. It influences the quality and costs of product. Highest curd yield was in BB genotype group – 28.5 ± 3.26%, while the lowest was observed in AA genotype group ($p < 0.05$). Ng-Kwai-Hang and co-authors (1984) have confirmed that milk obtained from cows with BB genotype had the highest curd yield compared to the milk collected from cows with AA and AB κ-casein genotype Curd yield can be explained by the difference in cows' genotype i.e. cows with AA genotype have a higher proportion of large casein micelles (average 206 nm), which reduces the curd yield efficiency and it is the explanation of lower curd yield. Burbano and collages (2010) have also found that the highest curd yield was from cows with BB κ-casein genotype, the average curd yield was observed in AB genotype, while the lowest curd yield was obtained from cows with AA genotype.

Curd formation time and its firmness plays an important role in the cheese production process. Significant differences were not found in curd firmness, however by analysing individual samples we have found that the highest curd firmness was in AA and AB genotype group (3.22 N in both group).

**Table 2.** Milk productivity and milk coagulation properties by κ-casein genotype

| Trait | AA ($n$ = 52) | AB ($n$ = 40) | BB ($n$ = 3) |
|---|---|---|---|
| Milk yield, kg | 19.30 ± 0.86 | 18.40 ± 1.25 | 17.10 ± 3.61 |
| Fat content, % | 4.26 ± 0.40$^{a}$ | 3.61 ± 0.22$^{b}$ | 4.07 ± 0.61 |
| Protein content, % | 3.39 ± 0.15 | 3.46 ± 0.83 | 3.47 ± 0.24 |
| Milk renneting time, min* | 18.47 ± 1.78$^{a}$ | 16.54 ± 2.06$^{ab}$ | 13.31 ± 1.84$^{b}$ |
| Curd yield, % | 21.10 ± 2.13$^{a}$ | 23.30 ± 1.15$^{ab}$ | 28.50 ± 3.26$^{b}$ |
| Curd firmness, N | 3.22 ± 0.50 | 3.22 ± 0.25 | 3.07 ± 0.72 |

$^{a,b}$ – traits with different letters in superscript are significantly different between breed ($p < 0.05$)
* data includes only coagulated samples ($n$ = 87)

The breed of dairy cows has considerably influenced the quality and yield of milk ASignificantly higher milk yield was observed in HBW group - 32.0 ± 2.99 kg, compared with LB and LZ groups (Table 3; $p < 0.05$).

Highest fat content was observed in LB group – 4.47 ± 0.13%, while lowest in HBW group 2.21 ± 0.20%, respectively. Fat content was significantly different between all groups ($p < 0.05$). Petrovska and Jonkus (2014) revealed that milk fat and protein content between LB genetic resources and HBW was not significantly different, whilst milk yield was significantly highest in HBW. However, Paura et al., (2012) have observed significantly higher milk fat content from LB compared with HBW breed in middle lactation stage ($p < 0.05$).

**Table 3.** Milk productivity and milk coagulation properties by breed

| Trait | Latvian Brown ($n$ = 56) | Latvian Blue ($n$ = 26) | Holstein Black and White ($n$ = 13) |
|---|---|---|---|
| Milk yield, kg | 19.10 ± 0.76$^{a}$ | 17.60 ± 1.32$^{a}$ | 32.00 ± 2.99$^{b}$ |
| Fat content, % | 4.47 ± 0.13$^{a}$ | 3.53 ± 0.23$^{b}$ | 2.91 ± 0.20$^{c}$ |
| Protein content, % | 3.45 ± 0.05 | 3.50 ± 0.09 | 3.43 ± 0.07 |
| Milk renneting time, min* | 16.86 ± 1.15$^{a}$ | 18.33 ± 3.19$^{a}$ | 27.17 ± 4.56$^{b}$ |
| Curd yield,% | 24.00 ± 0.79$^{a}$ | 22.50 ± 0.98$^{ab}$ | 18.70 ± 1.09$^{b}$ |
| Curd firmness, N | 3.21 ± 0.17 | 3.34 ± 0.22 | 2.64 ± 0.43 |

$^{a,b,c}$ – traits with different letters in superscript are significantly different between breed ($p < 0.05$)
* data about samples witch were coagulated ($n$ = 87)

Protein content of mil did not vary significantly among the analysed cow breeds Highest protein content was in LZ group – 3.50 ± 0.09%, while lower protein content in HBW breed. According to De March and co-authors breed affects milk fat and protein content significantly. Holstein is a high-yield breed that produces milk with significantly lower fat and protein content compared with local dual-purpose breeds such as Alpine Brown, Simmental, Alpine Gray (De Marchi et al., 2007).

Significantly different milk renneting time was observed in HBW breed – 27.17 ± 4.56 min. Favourable conditions for milk renneting positively affect curd formation, curd firmness, whey syneresis and cheese ripening processes (Cassandro et al., 2008). Shorter

renneting time was observed in LB (16.86 ± 1.15) and LZ (18.33 ± 3.19) breed cow's milk ($p < 0.05$). Varotto et al. (2014) have found that native dairy cow breeds produce milk with shorter milk renneting time comparing to HBW. Significantly higher curd yield (24.0 ± 0.79%) was observed in LB breed. Significant difference ($P < 0.05$) was established between HBW and LB breeds

Positive phenotypic correlation coefficient was observed between protein content and curd firmness and it has varied from 0.539 to 0.609. Similar tendency was found in Kübarsepp et al. (2005), where correlation coeffient of 0.310 was obtained. Negative correlation coeficient was present between protein content and milk renneting time (varieted from -0.490 to -0.510). Weak correlation was observed between milk renneting time and curd firmness (Fig. 1). Cassandro et al. (2008) found a strong phenotypic correlation (-0.76) between milk renneting time and curd firmness i.e. when milk renneting time decreases, curd firmness increses.

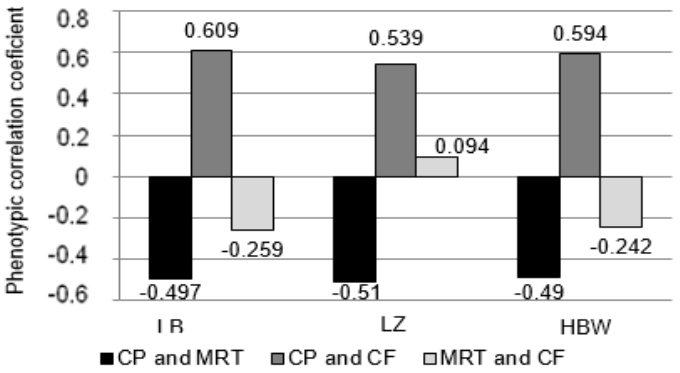



**Figure 1.** Phenotypic correlation coefiecients between CP and MRT (protein content, % and milk renneting time, min), CP and CF (protein content,% and curd firmness, N), MRT and CF (milk renneting time, min and curd firmness, N).

According to the previous research from Ostersen and co-authors (1997), milk coagulation properties are affected by different factors such as milk quality, breed, season, and herd. Milk chemical composition and coagulation properties were also significantly affected by stage of lactation (Zendri et al., 2017). Therefore for better understanding and explanation of breed and genotypes potential for cheese making further studies on milk protein profiles and genetic variants of different protein fractions during different seasons should be done.

## CONCLUSIONS

AA κ-casein genotype was more widespread in LB breed, while AB genotype was with highest frequency observed in LZ breed. Highest values of milk yield and milk fat content were observed in AA genotype group. Highest protein content and better coagulation properties were found in BB genotype group.

Significantly lower milk yield was obtained from LB and LZ breed, while milk fat content was significantly lower in HBW breed.

Most favourable milk coagulation properties were detected in BB genotype – highest curd yield, curd firmness and shorter milk renneting time.

In order to define an appropriate breed and κ-casein genotype for cheese making, further researches that takes into consideration other factors which affect milk composition and coagulation properties needs to be done

ACKNOWLEDGEMENTS. The research is supported by State Research Program AgroBioRes 3 project (LIVESTOCK).

## REFERENCES

Azevedo, A.L.S., Nascimento, C.S., Steinberg, R.S., Carvalho, M.R.S., Peixoto, M.G.C.D., Teodoro, R.L., Verneque, R.S., Guimarães, S.E.F. & Machado, M.A. 2008. Genetic polymorphism of the kappa-casein gene in Brazilian cattle. *Genetics and Molecular Research* **7**, 623–630.

Bonvillani, A.G., Di Renzo, M.A. & Tiranti, I.N. 2010. Genetic polymorphism of milk protein loci in Argentinian Holstein cattle. *Genetics and Molecular Biology* **23**, 819–823.

Burbano, G.L.Z., Cabrera, Y.M.E., Portilla, C.E.S. & Galindo, C.Y.R. 2010. Kappa casein genotypes and curd yield in Holstein cows. *Revista Colombiana de Ciencias Pecuarias* **23**, 422–428.

Cassandro, M., Comin, A., Ojala, M., Dal Zotto, R., De Marchi, M., Gallo, L., Carnier, P. & Bittante, G. 2008. Genetic parameters of milk coagulation properties and their relationships with milk yield and quality traits in Italian Holstein cows. *Journal of Dairy Science* **91**, 371–376.

Choobini, Z.M, Shadkhast, M., Moshtaghi, H., Dehkordi, S.H. & Shahbazkia, H.R. 2014. Polymorphism of κ-casein gene in Iranian Holsteins. *Iran Journal of Biotechnology* **12**, 56–60.

Cielava, L., Petrovska, S., Jonkus, D. & Paura, L. 2015. The effect of different bloodness level on Latvian Brown cow productivity. In: *Proceedings of the 7th International Scientific Conference 'Rural Development 2015'* Kaunas, DOI: 10.15544/RD.2015.038

Davoli, R., Dall'Olio, S. & Russo V. 1990. Effect of k-casein genotype on the coagulation properties of milk. *Journal of Animal Breeding and Genetics* **107**, 458–464.

De March, M., Dal Zotto, R. Cassandro, M., Bittante, G. 2007. Milk coagulation ability of five dairy cattle breeds. *Journal of Dairy Science* **90**, 3986–3992.

Ikonen, T., Ahlfors, K., Kempe, R., Ojala, M. & Ruottinen, O. 1999. Genetic Parameters for the milk coagulation properties and prevalence of noncoagulating milk in Finnish dairy Cows. *Journal of Dairy Science* **82**, 205–214.

Ikonen, T., Morri, A., Tyrisevä, A.M., Ruottinen, O. & Ojala, M. 2004. Genetic and phenotypic correlations between milk coagulation properties, milk production traits, somatic cell count, casein content and pH of milk. *Journal of Dairy Science* **87**, 458–467.

Jõudu, I., Henno, M., Värv, S., Kaart, T., Kärt, O. & Kalamees, K. 2007. Milk protein genotypes and milk coagulation properties of Estonian Native cattle. *Agricultural and Food Science* **16**, 222–231.

Kübarsepp, I., Henno, M., Kaart, T., Pärna, E., Viinalass, H. & Sabre D. 2006. Frequencies of κ-Cn and β-Lg genetic variants among Estonian cattle breeds and their effect on the milk renneting properties. In: *8th World Congress on Genetics Applied to Livestock Production,* Belo Horizonte, (in Brazil).

Kübarsepp, I., Henno, M., Viinalass, H. & Sabre, D. 2005. Effect of κ-casein and β-lactoglobulin genotypes on the milk renneting coagulation properties. *Agronomy Research* **3**, 55–64.

Lien, S., Kantanen J., Olsaker, I., Holm, L.E., Eythorsddottir, E., Sandberg, K., Dalsgard, B. & Adalsteinsson, S. 1999. Comparision of milk protein allele frequencies in Nordic cattle breeds. *Animal Genetics* **30**, 85–91.

Macheboeuf, D., Coulon, J.B. & D'Hour, P. 1993. Effect of breed, protein genetic variants and feeding on cow's milk coagulation properties. *Journal of Dairy Research* **60**, 43–54.

Malchiodi, F., Cecchinato, A., Penasa, M., Cipolat-Gotet, C. & Bittante, G. 2014. Milk quality, coagulation properties, and curd firmness modeling of purebred Holsteins and first- and second-generation crossbred cows from Swedish Red, Montbéliarde, and Brown Swiss bulls. *Journal of Dairy Science* **97**, 4530–4541.

Morkūnienė, K., Baltrėnaitė, L., Puišytė, A., Bižienė, R., Pečiulaitienė, N., Makštutienė, N., Mišeikienė, R., Miceikienė, I. & Kerzienė S. 2016. Association of kappa casein polymorphism with milk yield and milk protein genomic values in cows reared in Lithuania. *Veterinarija ir Zootechnika (Vet Med Zoot)* **74**, 27–32.

Ng-Kwai-Hang, K.F., Hayes, J.F., Moxley, J.E. & Monardes, H.G. 1984. Association of genetic variants of casein and milk serum proteins in milk fat and protein production by dairy cattle. *Journal Dairy Science* **67**, 835–840.

Ostersen, S., Foldager, J. & Hermansen, J.E. 1997. Effects of stage of lactation, milk protein genotype and body condition at calving on protein composition and renneting properties of bovine milk. *Journal of Dairy Research* **64**, 207–219.

Pečiulaitienė, N., Miceikiene, I., Mišeikienė, R., Krasnopiorova, N. & Kriauzienė, J. 2007. Genetic factors influencing milk production traits in Lithuanian dairy cattle breeds. *Gyvulininkystė* **1**, 32–38.

Paura, L., Jonkus, D. & Ruska, D. 2012. Evaluation of the milk fat to protein ratio and fertility traits in Latvian Brown and Holstein dairy cows. *Acta Agriculturae Slovenica* **100**, 155–159.

Petrovska, S. & Jonkus D. 2014. Latvian brown local breed cows milk yield, compositionand dry matter intake analyse. *Agriculture&Foresty* **60**, 81–86.

Sitkowska, B., Neja, W., Milczewska, A., Mroczkowski, S. & Markowska, A. 2013. Milk protein polymorphisms and effect of herds on cows`milk composition. *Journal of Central European Agriculture* **14**, 78–90.

Smiltina, D. & Grislis Z. 2010. Analysis of kappa-casein (CSN3) alleles in Latvian Brown and Latvian Blue breed populations. In: *Proceedings of annual 16th scientific conference Research for Rural Development 2010*. Jelgava, pp. 71–74.

Tsiaras, A.M., Bargouli, G.G., Banos, G. & Boscos, C.M. 2005. Effect of kappa-casein and beta-lactoglobulin loci on milk production traits and reproductive performance of Holstein cows. *Journal of Dairy Science* **88**, 327–334.

Varotto, M., De Marchi, M., Penasa, M. & Cassandro, M. 2014. Milk coagulation ability of Rendena and Holstein-Friesian cattle breeds. *Acta Agraria Kaposváriensis* **18**, 89–95.

Velmala, R., Mantysaari, E. A., Maki-Tanila, A. 1995. Molecular genetic polymorphism at the k-casein and b-lactoglobulin loci in Finnish dairy bulls. *Journal of Agricultural Science in Finland* **26**, 431–435.

Zannoni, M. & Annibaldi, S. 1981. Standardization of the renneting ability of milk by Formagraph. *Scienza e Tecnica Lattiero-Casearia* **32**, 79–94.

Zepeda-Batista, J.L., Alarcón-Zúñiga, B., Ruíz-Flores, A., Núñez-Domínguez, R. & Ramírez-Valverde, R. 2014. Polymorphism of three milk protein genes in Mexican Jersey cattle. *Electronic Journal of Biotechnology* **18**, 1–4.

Zendri, F., Ramanzin, M., Cipolat-Gotet, C. & Sturaro, E. 2017. Variation of milk coagulation roperties, cheese yield, and nutrients recovery in curd of cows of different breeds before, during and after transhumance to highland summer pastures. *Journal of Dairy Research* **84**(1), 39–48.

*Agronomy Research* **15**(S2), 1419–1425, 2017

# Increasing the level of hydration of biopolymers in meat processing systems based on the use of acoustically activated brines

L. Tsirulnichenko[1,*], I. Potoroko[1], O. Krasulya[2], I. Gudina[1]

[1]South Ural State University, Higher School of Medicine and Biology, Department of Food and Biotechology, 85 Lenina Avenue, 454080, Chelyabinsk, Russia
[2]Moscow State University of Technologies and Management named after K.G. Razumovskiy, Department of regulation of raw food products and examination of goods, 73, Zemlyanoy Val, 109004, Moscow, Russia
*Correspondence: linchikz@mail.ru

**Abstract**. The study aims to develop effective ways to improve the level of protein hydration of poultry. We studied the chemistry of the process of moisture binding by minced meat produced from pectoral and femoral muscles of broiler chickens. The reference brines were prepared from potable water and the test brines were sonicated. The samples which were held for a predetermined time and evaluated for structural integrity and heat treatment losses. The parameter values shown in the experimental session turned out to be higher in comparison with that shown in the reference session. A positive effect of ultrasound on the increase of hydration properties of poultry proteins and reduction of brine treatment time by more than 2 times and reduction of losses during heat treatment by 10% were recorded.

**Keywords:** ultrasonic treatment, meat processing technology, cavitation, hydration

## INTRODUCTION

Hydration (from Greek hydor 'water') usually refers to the process of binding water with chemicals. Dehydration refers to the process, which is reverse to hydration, i.e. the loss of water bound by substances. Meat processors, especially those working with frozen raw meat, can observe that defrosting is accompanied by the process reverse to hydration, the control over which has a significant importance. Modern science dealing with food production pays much attention to solving the problem of safe and maximum moisture binding.

Currently, poultry is one of the most commonly consumed meat among all types of meat worldwide. Quality characteristics of poultry meat are influenced by many factors: genes (bird species, breed, cross, age), feed (ration type, nutrient balance), technology (breeding method, cramming duration, management conditions, preslaughter treatment, slaughter and processing), etc. (Shapiro & Mercier, 1994).

Therefore, it is quite difficult to ensure stable quality of finished poultry products. This is due to the high variability in the quality of raw meat in terms of chemical composition and the wide spread of PSE (Pale, Soft and Exudative) and DFD (Dark,

Firm and Dry) meat observed when raw meat has low moisture binding ability or poorly retains moisture during processing, especially at the brine treatment stage. Such problems for food technologist are associated with the risk of rejects. Therefore, producers resort to a variety of functional additives, for example, the use of phosphates which help retain moisture during brine treatment and make meat succulent. However, food safety in this case becomes compromised.

One of the most promising and alternative ways to increase the water-binding capacity and thermal resistance of raw meat biopolymers is the use of acoustically activated brines (Povey, 1998). Currently, the food industry developed methods for preparing aqueous solutions of electrolytes with the use of ultrasonic cavitation (Shestakov & Babak, 2012). The whole process comes down to primary ultrasonic treatment of water followed by mixing it with other components of the brine (Pilipenko et al., 2012). Electrolyte ions in such water acquire dense solvate shells made of free water molecules, i.e., the ions are immobilized by them which prevents their association. As a result, mass-exchanging and hydration processes are intensified during brine treatment of meat products (Shestakov et al., 2011).

Studies of the use of activated liquids in the meat industry defined the main directions of their use in salted meat production. It was found that the use of brines based on electro-activated water in the salted meat production contributes to a more uniform distribution of brine ingredients and speeds up the physico-chemical and biochemical processes occurring during the brine treatment of meat. Of special interest are the biopolymer hydration ability and a pronounced bactericidal effect of such cavitation-activated media.

These effects formed the basis of a working hypothesis about the possibility of increasing the thermal resistance of raw meat biopolymers using acoustic vibrations in the preparation of brines injected into the raw meat.

The goal of this work is to study the applicability of acoustic vibrations generated by ultrasound to increase the level of hydration of poultry biopolymers.

## MATERIALS AND METHODS

### Raw poultry

Model samples of broiler chicken minced meat were used in the study. We used broiler chicken minced meat of different thermal state (chilled and defrosted) with different morphological characteristics: on the basis of breast (‘white’ meat) and thigh (‘red’ meat) muscles, their 1:1 mixture (‘mixture’).

Broiler chicken meat (dressed chicken) used in the preparation of test and reference samples demonstrated the following properties: the protein mass fraction varied from $16 \pm 0.4$ to $20 \pm 0.6$%; the fat mass fraction from $10 \pm 0.2$ to $16 \pm 0.4$%, and pH from 5.4 to 6.4.

We studied the broiler chicken meat produced by Russian poultry complexes in the Chelyabinsk, Kurgan, Sverdlovsk and Tyumen regions. We selected 100 samples from each batch, and repeated the survey at least 15 times.

### Minced meat model sample (emulsion) preparation method

The meat was ground through a 2.5 mm grate. The treated table salt solution (2.5%) was added to the treated meat. All samples were divided into two batches: for reference

and test. For the preparation of reference samples we used tap water-based brines, and the test batch – water sonicated with an ultrasound technological device UZTA-0.4/22-OM (Volna, Russia). The amount of added brine was 30% by weight of the raw meat.

**Study procedure**

The treated minced meat model samples were held for a predetermined time and monitored for changes in shear viscosity and thermal treatment losses. The experiment was performed at temperatures close to the process. Shear viscosity was measured with a rheometer Brookfield DV-III Ultra. Heat treatment 'losses were determined' by weighing.

Mathematical measurement processing was carried out by conventional variational statistics methods and expressed as the arithmetic mean (M) and its standard error (m). We used the Mann – Whitney test (U) to determine the statistically significant differences between the test and reference groups. Differences were considered significant at $p < 0.05$. Statistical interrelations were studied using nonparametric correlation analysis by calculating the Spearman correlation coefficients (Rs).

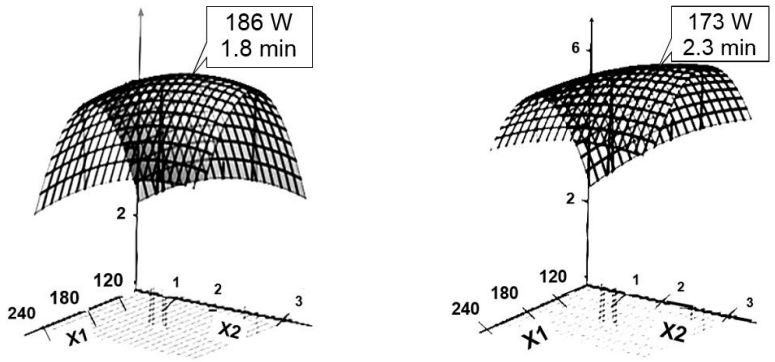
## RESULTS AND DISCUSSION

Raw meat brine treatment is very closely related to the process of hydration of meat biopolymers. Minced meats are emulsions, i.e. dispersions comprising dispersed medium – water – and dispersed phase – particles of the crushed missela muscular tissues and macromolecules. According to Rebinder's (1979) classification, meat emulsions refer to coagulation emulsions, where a solid carcass is formed in the hydration of the dispersed phase resulting in the advanced structural and mechanical properties of the finished products. Therefore, their quality is directly dependent on the amount and form of moisture binding, primarily with the protein components of minced meat. As a rheological property, dynamic viscosity most fully characterizes the course of the hydration process in the mechanical treatment of minced meat.

Acoustic vibrations in water generate enormous pressure pulses in the water which, correspondingly, cause deformations travelling in the water at the speed of sound. The transformation of the potential energy of such deformations implements an above-thermal mechanism of destruction of molecular associates whereby only water passes into a thermodynamically non-equilibrium state, and salt dissolved therein completely dissociates into ions, which will be immobilized by polar monomolecular water or tightly bound in the forming protein solvate shells.

Let us consider an example of the effect of activated brines on the rheology of meat emulsions below.

A two-way analysis was conducted to determine the optimal mode for acoustic influence of ultrasound based on the thermal condition of the meat. We selected power and duration of ultrasonic treatment as variables. Monitored parameter: level of hydration (content of moisture in the product after thermal treatment) on Fig. 1.




a b

**Figure 1.** Dependence of the level of protein hydration of poultry on the power and duration of ultrasonic treatment for minced meats from chilled (a) and defrosted (b) raw meat ($X_1$ – power, W, $X_2$ – time, min).

Hence, salt brines injected into the test samples were treated under the following conditions – frequency 22 ± 1.65 kHz, power 186 W, exposure 1.8 min for minced meat from chilled raw meat and power 173 W, exposure 2.3 min for defrosted raw meat.

Results of the study of the shear viscosity of minced poultry are shown on Figs 2 and 3.

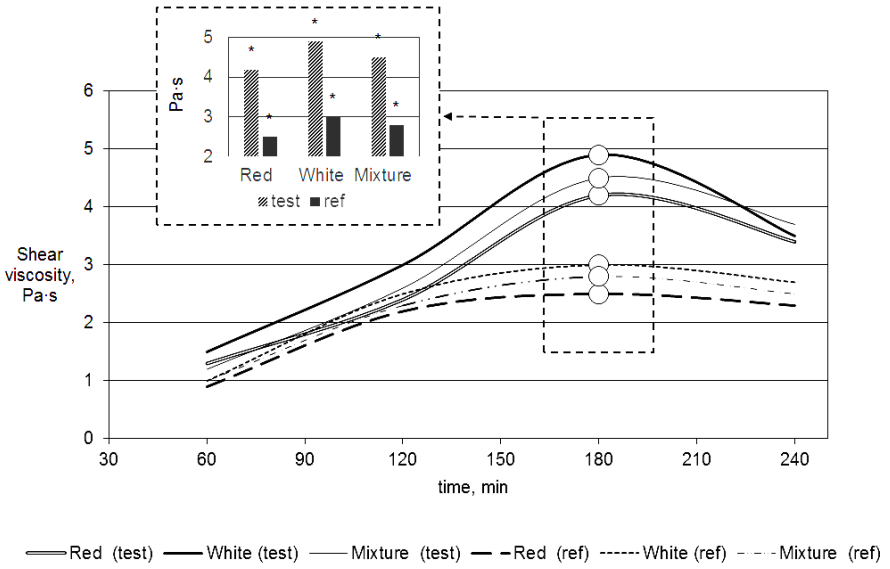



**Figure 2.** Shear viscosity of minced meats from chilled broiler chicken meat. (deviation does not exceed 0.3 for all indicators).

Gorbatov (1979) established the structure tightening period for thinly minced meats: after 2–4 hours of holding at 22 °C minced meats demonstrate the highest

structural and mechanical characteristics due to the thixotropic tightening of their structure.

Tightening of minced meat structure is observed after 180 minutes of holding. The test samples showed higher shear viscosity relative to the reference samples, i.e., muscle fibers saturate with moisture more fully and particles swell better.

The highest viscosity values were registered for chilled raw meat: white meat – 4.8 Pa·s; red meat – 3.7 Pa·s; mixture – 4 Pa·s (averaged values of viscosity). The obtained dynamic viscosity distribution values depends on the chemical composition of muscle development: The fat content in thigh muscles (red meat) is greater than that in pectoral muscles (white meat) resulting in the increase of the surface tension at the water-tissue interface and decrease in the biomass wettability, which ultimately determines the decrease in viscosity.

Defrosted test samples demonstrated high viscosity values, which allows us to speak about the normal course of the process of saturation of biomass with moisture and its retention in the meat system, and solve the problem of low technological properties of defrosted raw meat.

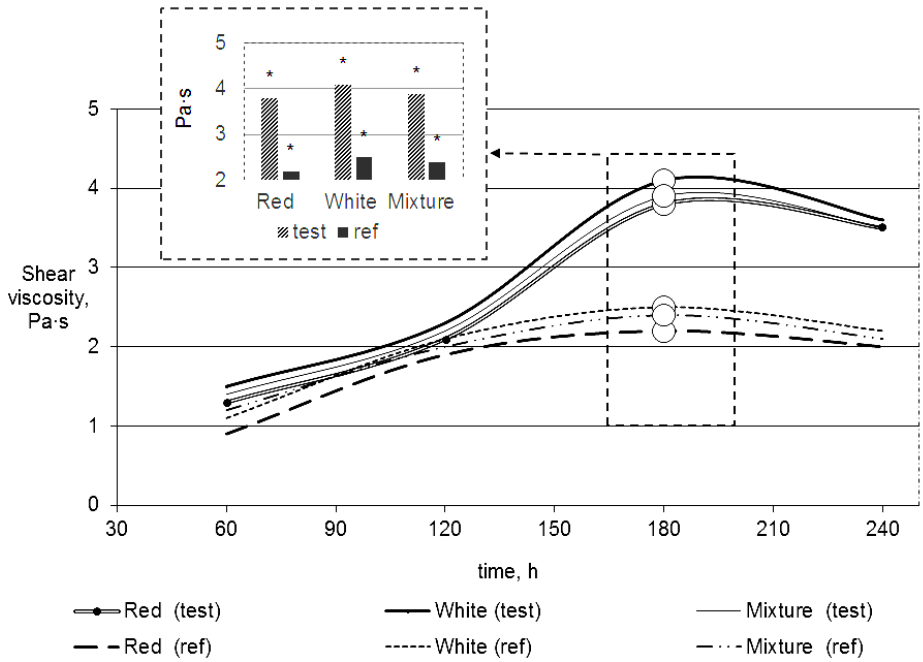



**Figure 3.** Shear viscosity of minced meats from defrosted broiler chicken meat (deviation does not exceed 0.3 for all indicators).

It should also be noted that the reference samples of minced meats are resistant to increased temperatures and prolonged mechanical stress – in the final phase of the experiment: no sharp decrease in the structural and mechanical properties (viscosity) was observed. At the same time we noted a sharp decrease in the shear viscosity trend of the reference samples which was caused by grinding of the remaining muscle fibers and connective tissue and changes in moisture bond forms.

Similar processes can be observed during chopping: minced meat particles must be bound with such amount of water which turns them into a homogeneous mass.

The positive trend in the structural and mechanical characteristics is consistent with positive changes in water-holding capacity observed in the test samples of coarse minced meat based on breast muscles of chilled poultry.

In the sausage production technology holding of coarse minced meats before chopping may take up to 24 hours for the purpose of deeper penetration of brine components.

We have found that acoustic activation has a beneficial effect on the holding time of coarse minced meat before chopping (Fig. 4).

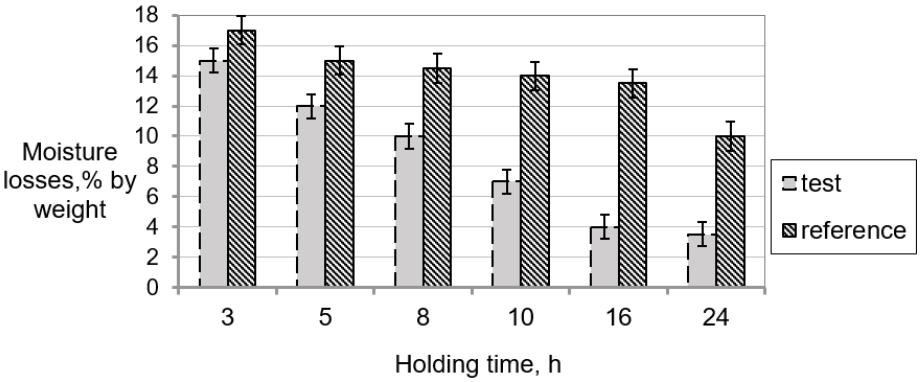



**Figure 4.** Dependence of the amount of thermal treatment losses of minced meats on the holding time. Statistically significant differences ($p < 0.05$) between test and reference samples by the Kruskal-Wallis criteria.

The test samples have specified characteristics as early as after 3 hours of holding, thereby reducing the time required by 2 times. For the test samples moisture losses during thermal treatment amounted to 10%, which is 5% lower than the reference samples. We noticed that lower moisture loss increased the yield of finished products by 5%. Using calculation methods we found that the acoustic effects of ultrasound on the brine can reduce the amount of water-retaining additives injected as per formula by up to 75%, shorten the maturing period by 2 times on average.

## CONCLUSIONS

Acoustic vibrations generated by ultrasound increase the level of hydration of poultry myofibrillar proteins.

Acoustic activation of liquid brine media improves the functional and process properties of poultry. This will retain enough moisture in the finished meat products and add extra juiciness.

It was established in the course of the study that the application of brines leads to the reduction of time required to hold minced meat, and that translates directly into intensification of the technological process and reduce the risk of defects.

ACKNOWLEDGEMENTS. This article was written with support from the government of the RF (Resolution No 211 of 16.03.2013), Agreement No 02.A03.21.0011, and subsidies for the fulfilment of a fundamental part of a state order, Project No 40.8095.2017/BCh. The work was also supported by Act 211 of the government of the Russian Federation, Contract No 02.A03.21.0011, and was carried out with the financial support of the UMNIK Programme's Assistance Fund.

## REFERENCES

Ashokkumar, M., Lee, J., Zisu, B., Bhaskarcharya, R., Palmer, M. & Kentish, S. (2009). Hot topic: Sonication increases the heat stability of whey proteins. *Journal of Dairy Science* **92**, 5353–5356.

Brotchie, A., Grieser, F. & Ashokkumar, M. 2009. Effect of power and frequency on bubble-size distributions in acoustic cavitation. *Physical Review Letters* **102**, art. no. 084302.

Chandrapala, J., Oliver, C., Kentish, S. & Ashokkumar, M. 2012. Ultrasonics in food processing. *Ultrasonics Sonochemistry* **19**, 975–983.

Gorbatov A.V. 1979. *A rheology of meat and milk*. Publishing house 'Food promyshelnnost', 382 pp. (in Russian).

Kentish, S., Wooster, T.J., Ashokkumar, M., Balachandran, S., Mawson, R. & Simons, L. (2008). The use of ultrasonics for nanoemulsion preparation. *Innovative Food Science and Emerging Technologies* **9**, 170–175.

Krasulya, O., Shestakov, S., Bogush, V., Potoroko, I., Cherepanov, P. & Krasulya, B. 2014. Applications of sonochemistry in Russian food processing industry. *Ultrasonics Sonochemistry* **21**, 2112–2116.

Pilipenko, T., Pilipenko, N. & Potoroko, I. (2012). Use of electrophysical methods by production and quality control of foodstuff. *The Commodity Researcher of Foodstuff* **4**, 33.

Porova, N., Botvinnikova, V., Krasulya, O., Cherepanov, P. & Potoroko, I. 2014. Effect of ultrasonic treatment on heavy metal decontamination in milk. *Ultrasonics Sonochemistry* **21**, 2107–2111.

Rebinder P.A. 1979. *The superficial phenomena in disperse systems. Physical and chemical mechanics. Chosen works*. Publishing house 'Science', Moscow, 384 pp. (in Russian).

Povey, M.J.W. 1998. Ultrasonics of foodə. *Contemporary Physics* **39**, 467–478.

Shanmugam, A. & Ashokkumar, M. 2015. Characterization of ultrasonically prepared flaxseed oil enriched beverage/carrot juice emulsions and process-induced changes to the functional properties of carrot juice. *Food and Bioprocess Technology* **8**, 1258–1266.

Shapiro, A. & Mercier, C. 1994. Safe food manufacturing. *Science of the Total Environment* **143**, 75–92.

Shestakov, S., Krasulya, O., Artemova, J. & Tikhomirova, N. 2011. Ultrasonic water treatment. *Dairy Industry* **5**, 39–42.

Shestakov S. & Babak V. 2012. Mathematical Model of the Spatial Distributing of Density of Erosive Power of Multibubble Cavitation. *Applied Physics Research* **4**, 64–77.

Truhaut, R. 1991. Toxicology, social science and the humanitarian. *Bulletin de l'Academie Nationale de Medecine* **175**, 1385–1402.

*Agronomy Research* **15**(S2), 1426–1434, 2017

# Selected wastewater parameters from the vegetable washing process

P. Vaculik[1,*], A. Smejtkova[1], M. Prikryl[1], O. Drabek[2], Z. Votruba[1] and L. Lexa[1]

[1] Czech University of Life Sciences Prague, Faculty of Engineering, Department of Building Technological Equipment, Kamýcká 129, 165 21 Prague 6-Suchdol, Czech Republic
[2] Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Soil Science and Soil Protection, Kamýcká 129, 165 21 Prague 6-Suchdol, Czech Republic
* Correspondence: vaculik@tf.czu.cz

**Abstract.** This article follows selected parameters in wastewater which arise from the washing process for root vegetables, which is one of those problems which are current in terms of water usage. With a growing population, industrialisation, and urban development, there is also a growing demand for water resources. Industries which are dealing with the processing of agricultural products and food production in general significantly contribute to the growing consumption of water. Technology which is used for cleaning vegetables also significantly affect this growth in water consumption. Increasing demands on the quality of vegetables (eg. the cleanliness of vegetables at the point of sale), also leads to the necessity for more effective post-harvest cleaning, something which is carried out both with dry and wet methods. This article examines the cleaning process for selected root vegetables, particularly carrots and potatoes, by determining selected properties of the output process water in an assessed technological line. This line is specific with regard to its methods for cleaning carrots and potatoes. Following the investigation, the line was assessed as being satisfactory with respect to the quality of the input and output water. The monitored parameters of the process water (eg. concentrations of selected elements in the process water and concentrations of selected inorganic anions in the process water, mainly Na and Pb) from cleaning carrots and potatoes were considered as being satisfactory for recirculation into the cleaning process and therefore a reduction was achieved in overall water consumption.

**Key words:** food industry, technological line, vegetables, washing vegetables, wastewater

## INTRODUCTION

Water is defined as an essential component of the environment. Without it life cannot exist. Given the irreplaceable nature of water, the wastewater treatment process is one of the most important conditions for sustainable development. As with energy saving (Vaculík et al, 2013), due to ongoing and further expected climate-related changes, it can be assumed that water management and water treatment will increasingly be an issue that will be have to be addressed (Alsiņa et al, 2016; Borys & Küüt, 2016). Vegetables are an important part of our diet (Dubrovskis & Plume, 2015). A proportion

of the production reaches consumers in a fresh state, and a proportion of it is processed into other products.

Fig. 1 shows the general scheme involved in the product handling process, from its harvesting in the field to its distribution, either for direct consumption or further processing. Input and output variables such as consumed energy are assigned to each operations sequence (Kern, 2006). Table 1 shows the parameters for selected areas of the lines.

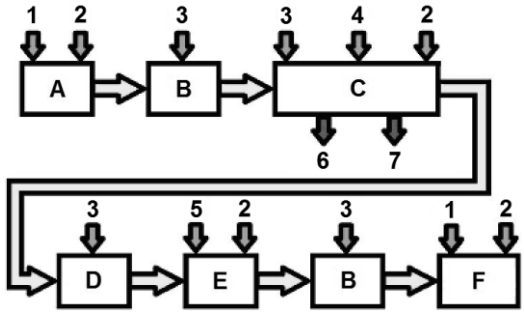



**Figure 1.** General scheme showing the technological line for cleaning vegetables. Explanatory notes: A – product transportation from the field, B – weight measuring, C – post-harvest treatment, D – storage, E – loading, F – transportation to the customer, 1 – fuel, 2 – human activity, 3 – electrical energy, 4 – water, 5 – power (forklifts), 6 – polluted water, 7 – organic waste.

Post-harvest treatment most often occurs in three stages. Step one involves cleaning the vegetables, where the aim is to remove organic and inorganic substances from their surfaces. This process is generally carried out under dry conditions or using water. Under dry conditions, coarse impurities, stems and such like are removed from the product. This is followed by washing, in particular for root vegetables and cucumber, which removes the remaining surface dirt.

**Table 1.** The parameters for selected areas of the lines.

| **The performance of facility** | | | | |
|---|---|---|---|---|
| De-stoning Machine (ton $hr^{-1}$) | Soaking Reservoir (ton $hr^{-1}$) | Machine for remove leaves (ton $hr^{-1}$) | Brush Polisher (ton $hr^{-1}$) | Interoperation Container ($m^3$) |
| 20 | 5-30 | 15 | 8-20 | 19 |
| **The overall performance of the process line** (ton $day^{-1}$) | | | | |
| Potatoes | | | | 312 |
| Carrots | | | | 60 |

Depending upon the type of vegetable being processed and the degree of contamination, the following four types of washing equipment are most frequently used: (i) soak-wash equipment: the product is soaked in this equipment, which releases the deposited impurities. The product is then usually moved by conveyor from the soak-wash equipment to the primary washing equipment; (ii) shower washing equipment: washing is carried out using strong water pressure (0.8–1.2 MPa) from the shower

component in the tunnel equipment. Nozzles are positioned above and below the conveyor which contains the product. An alternative is a mesh rotating grate. A shower device is often positioned above the conveyor, and the device removes soaked-off impurities from the product; (iii) drum washing equipment: the principle of this form of washing equipment is a partially submerged perforated rotating drum. The product is cleaned via mutual friction between the products and the drum's surface. The product can also be rinsed thanks to the immersion of the drum in water; (iv) brush washing equipment: the main mechanisms of this type of washing equipment involves rotating shafts which are fitted with brushes of different levels of hardness according to the product being washed. This is the most common type of washing equipment.

During post-harvest treatment, the second step is quality sorting. The aim is to remove pieces that do not meet quality requirements in terms of their appearance, colour, or other defects. Sorting takes place on inspection belts which carry the product at a speed of between 20 to 40 cm $s^{-1}$ and also ensure that the product is rotated. Workers remove unsuitable vegetables. Vegetables can also be sorted using optical sorting equipment. In most cases, a desk is placed behind such sorting equipment with additional staff available to provide assistance.

The third step in post-harvest treatment is size sorting. Three structural systems are used for the purpose of sorting, these being rotary slit sorters, longitudinal slit sorters, and sorters equipped, with a system of screens with unequally-sized holes being employed.

A large quantity of water is used during the post-harvest treatment of vegetables, on whose cleanliness ever greater demands are being placed during the sale of such vegetables (Simons & Sanquansri, 1997). Both from an economic and ecological perspective, it is desirable to decrease the amount of water being used. One method could be the repeated use of the water, but the water must contain the corresponding parameters. This article deals with this problem (Goliáš, 2014; Pao et al, 2012).

## MATERIAL AND METHODS

An analysis of wastewater from the line for the post-harvest treatment of potatoes and carrots was carried out at a company which is located in the Central Bohemian Region of the Czech Republic (concerning an important and representative agricultural company). The company's activities consist mainly of the cultivation and post-harvest treatment of vegetables (mainly involving carrots and potatoes).

Water which is intended for washing vegetables in a drum washer is pumped from a tank with a volume of 30$m^3$ (the water is provided via a water pipeline). The tank is drained three times during the day. The daily water consumption for both lines (ie. the line for potato processing and the line for carrot processing) is therefore approximately 90$m^3$ of water (the average efficiency rates for this line is between ten and fifteen tons of vegetables an hour). The water temperature is about 10°C. After passing through the lines, the water contains mainly impurities in the form of dirt, sand, and organic vegetable residues such as carrot chips, stems, etc. Using gravity, the polluted water flows to a sedimentation tank, Nos 1 or 2 (see Fig. 2). Only one of the two tanks is always used. During this time the unused tank is cleaned of any deposits. The use of tanks Nos 1 and 2 alternates in monthly intervals, and approximately 330$m^3$ of impurities settle in the tank during this time. From these outer tanks, in which the water remains

for approximately four days, the water overflows through the outflow into the intermediate tank, No 3. These sedimentation tanks are not able to separate undissolved substances as efficiently and at the required levels of quality so that the water can be returned to the line. The water from the middle tank is therefore pumped out using three sludge pumps so that it reaches the recipient in accordance with Decree No 401/2015 Coll (Nařízení vlády č. 401/2015 Sb, 2015).

Samples of wastewater were taken over the course of three months. Measurements were carried out, with Sample A being taken in November 2015, Sample B in December 2015, and Sample C in January 2016.

Three sampling points were chosen (see Fig. 2), on the inflow to the sedimentation tanks (1), and to the overflow between the tanks (2), with the last sampling point being at the pumps at which water is removed from the tank and delivered to the recipient (3). The samples were removed at each collection point during a precisely-defined period of time while all of the water was drained from the line (the majority of the polluted water arrives at this time), during the process of draining the water from the pre-soak tub, and the third during normal outflow from the lines. A mixed sample was created from these three samples through their being mixed together. Three composite samples divided according to collection points were therefore obtained in one day.

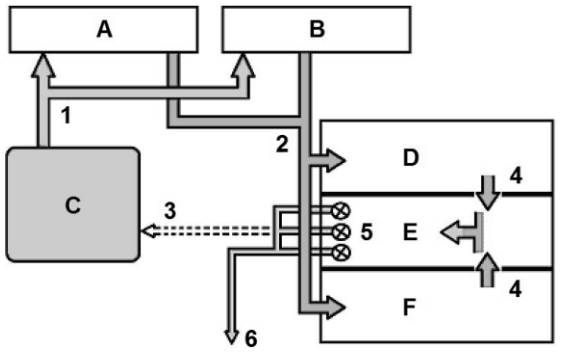



**Figure 2.** Block diagram showing the flow of water during the waste management of the monitored line. Explanatory notes: A – technological line for processing carrots, B – technological line for processing potatoes, C – reservoir with pure water, D – settling tank No 1, E – settling tank No 3, F – settling tank number 2, 1 – clean water, 2 – polluted water, 3 – pipelines from the settling tank to reservoir, 4 – water overflow, 5 – water pumps, 7 – brook.

Those components which are present in a given sample can be divided into undissolved and dissolved substances according to their physical properties. All of the substances are obtained by the sum of these two values. In terms of wastewater treatment, these values serve as indicators for the basic chemical composition of the water (the COD and BOD and microbiological quality were all determined within the context of another research project). Gravimetric determination is used to determine these values.

**The gravimetric determination of all substances** (TS)

A sample of wastewater that is properly homogenised is first evaporated and is then dried at 105 °C to a constant weight (dry matter). The process of annealing dry matter at

550 °C is carried out in order to determine the organic and inorganic proportions in the sample. The difference in the weight of the dry matter and annealed dry matter is labelled as the annealing loss. The annealing loss is the amount of substances in dry matter that volatilise or burn at temperatures of up to 550 °C (organic substances - organic TS). Minerals are largely resistant up to this temperature (inorganic substances - inorganic TS).

**The gravimetric determination of dissolved substances** (TDS)

Dissolved substances are labelled as having passed through the filtration device with an average pore size of 0.45 μm. The acquired filtrate is dried and annealed in the same way as for the determination process for all substances. The values of all organic and inorganic dissolved substances are obtained (TDS, organic DS, and inorganic DS).

**The gravimetric determination of undissolved substances** (TSS)

Undissolved substances which are contained in a sample are trapped on glass fibres in the filter. The acquired substances are dried and annealed in the same way as for the process which covers the determination for all substances. The values for all organic and inorganic undissolved substances are obtained (TSS, organic SS, and inorganic SS) (Horáková, 2003).

In addition to dissolved substances, an element analysis was conducted of the collected samples of wastewater.

The solution was filtered through 0.45μm Nylon disk filters (Cronus, UK) prior to analysis. The contents of the selected elements (elements which were monitored by decree - Vyhláška č. 252/2004 Sb and Council Directive 98/83/EC (As, B, Be, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Na, Ni, Pb, Sb, Al)) were determined by means of ICP-OES (DUO iCap 7000, Thermo Scientific) under standard analytical conditions. The quality of the analysis was controlled using blanks and the standard reference materials (Ash et al, 2016). Moreover, after filtration through a 0.45 μm nylon membrane filter, concentrations of mayor inorganic anions (F, Cl, $NO_2$, $NO_3$, $(SO_4)_2$ and pH) in the washing water were determined by means of ion-exchange chromatography with suppressed conductivity. An anion chromatograph ICS 1600 was used (Dionex, USA), equipped with an IonPac AS11-HC guard (Dionex, USA), and analytical columns. The eluent composition was 1–37.5 mM KOH with a gradient of 1–50 mins; and the flow rate was set to 1 mL $min^{-1}$. In order to suppress eluent conductivity, an ASRS 300–4 mm suppressor (Dionex, USA) and a Carbonate Removal Device 200 (Dionex, USA) were used (Mercl et al, 2016).

## RESULTS AND DISCUSSION

Table 3 shows the measured values for all substances, including dissolved substances and undissolved substances (Table 2 shows the measured values for the process water parameters).

These substances are further subdivided into organic and inorganic. It is mainly the A-3, B-3, and C-3 values which are important, as they characterise the water at the outlet, ie. water which is potentially intended for repeated use. When compared to the values indicated for tanks Nos 1 and 2, the cleaning ability of sedimentation tanks can be

evaluated. Each sample (A-1, A-2 to C-3) is mixed. This means that the sample contains water of ten subscriptions.

**Table 2.** The parameters for process water

| Concentrations of selected elements in the process water | | | | |
|---|---|---|---|---|
| **Al** (mg $l^{-1}$) | **As** (mg $l^{-1}$) | **B** (mg $l^{-1}$) | **Be** (mg $l^{-1}$) | **Ca** (mg $l^{-1}$) |
| 0.02 | 0.00 | 0.01 | 0.00 | 0.01 |
| **Cd** (mg $l^{-1}$) | **Cr** (mg $l^{-1}$) | **Cu** (mg $l^{-1}$) | **Fe** (mg $l^{-1}$) | **Mg** (mg $l^{-1}$) |
| 0.00 | 0.00 | 0.00 | 0.01 | 0.00 |
| **Mn** (mg $l^{-1}$) | **Na** (mg $l^{-1}$) | **Ni** (mg $l^{-1}$) | **Pb** (mg $l^{-1}$) | **Sb** (mg $l^{-1}$) |
| 0.02 | 80.00 | 0.01 | 0.01 | 0.00 |
| Concentrations of selected inorganic anions in process water | | | | |
| **F** (mg $l^{-1}$) | **Cl** (mg $l^{-1}$) | **$NO_2$-** (mg $l^{-1}$) | **$NO_3$-** (mg $l^{-1}$) | **$SO_4$ 2-** (mg $l^{-1}$) |
| 0.37 | 57.24 | 0.03 | 0.18 | 205.45 |

Table 3 shows that the sedimentation tanks primarily catch undissolved substances. At the inlet for the tank, the concentration of all undissolved substances ranges between 0.19–9.63g $l^{-1}$, while at the outlet the range varies between 0.05–0.52g $l^{-1}$.

**Table 3.** Measured values using gravimetric methods

| Sample | **TS** (g $l^{-1}$) | **TDS** (g $l^{-1}$) | **TSS** (g $l^{-1}$) | **VS** (g $l^{-1}$) | **org.DS** (g $l^{-1}$) | **org.SS** (g $l^{-1}$) | **inorg.TS** (g $l^{-1}$) | **inorg.DS** (g $l^{-1}$) | **inorg.SS** (g $l^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| A-1 | 5.96 | 1.53 | 4.43 | 0.8 | 0.32 | 0.48 | 5.16 | 1.21 | 3.95 |
| A-2 | 2.13 | 1.49 | 0.64 | 0.45 | 0.29 | 0.16 | 1.68 | 1.20 | 0.48 |
| A-3 | 1.42 | 1.37 | 0.05 | 0.27 | 0.24 | 0.03 | 1.15 | 1.13 | 0.02 |
| B-1 | 1.70 | 1.51 | 0.19 | 0.42 | 0.32 | 0.10 | 1.28 | 1.19 | 0.09 |
| B-2 | 1.52 | 1.44 | 0.08 | 0.28 | 0.27 | 0.01 | 1.24 | 1.17 | 0.07 |
| B-3 | 1.77 | 1.42 | 0.35 | 0.31 | 0.24 | 0.07 | 1.46 | 1.18 | 0.28 |
| C-1 | 11.28 | 1.65 | 9.63 | 1.57 | 0.33 | 1.24 | 9.71 | 1.32 | 8.39 |
| C-2 | 2.04 | 1.71 | 0.33 | 0.39 | 0.38 | 0.01 | 1.65 | 1.33 | 0.32 |
| C-3 | 1.88 | 1.36 | 0.52 | 0.39 | 0.28 | 0.11 | 1.49 | 1.08 | 0.41 |

Explanatory notes: TS – all substances, TDS – dissolved substances, TSS – undissolved substances, VS – organic all substances, org.DS – organic dissolved substances, org. SS – organic undissolved substances, inorg. TS – inorganic all substances, inorg. DS – inorganic dissolved substances, inorg. SS – inorganic undissolved substances.

In Sample B (December 2015), the lower volume of TS is probably caused by different soil moisture levels which closely correlate to the weather conditions. Consequently, some specific parameters which are presented in Tables 3, 4, and 5 exhibit significantly different values. We can also expect the amount of clay and soil organic matter, together with the soil moisture, to have a great deal of influence on the volume of TS if different study plots are to be investigated.

Table 4 shows the concentrations of selected elements in the wastewater. The limit values for individual elements were drawn from Decree No 252/2004 Coll (Vyhláška č. 252/2004 Sb, 2004), which determines the hygienic requirements for drinking water and hot water, and the frequency and scope of drinking water controls.

**Table 4.** Concentrations of selected elements in the wastewater

| Sample | **Al** 396.152 {85} (Radial) (mg $l^{-1}$) | **As** 189.042 {478} (Axial) (mg $l^{-1}$) | **B** 208.959 {461} (Axial) (mg $l^{-1}$) | **Be** 234.861 {143} (Axial) (mg $l^{-1}$) | **Ca** 396.847 {85} (Radial) (mg $l^{-1}$) |
|---|---|---|---|---|---|
| DL | 0.0386 | 0.0068 | 0.0068 | 0.0068 | 0.0079 |
| A-1 | n.d. | n.d. | 0.15 | n.d. | > 100 |
| A-2 | 0.06 | n.d. | 0.16 | n.d. | > 100 |
| A-3 | n.d. | n.d. | 0.15 | n.d. | > 100 |
| B-1 | n.d. | n.d. | 0.14 | n.d. | > 100 |
| B-2 | n.d. | n.d. | 0.14 | n.d. | > 100 |
| B-3 | n.d. | n.d. | 0.16 | n.d. | > 100 |
| C-1 | n.d. | n.d. | 0.01 | n.d. | > 100 |
| C-2 | 0.08 | n.d. | 0.01 | n.d. | > 100 |
| C-3 | 0.11 | n.d. | 0.01 | n.d. | > 100 |
| Limit values | 0.20 | 0.01 | 1.00 | 0.0020 | 30.00 |
| Sample | **Cd** 226.502 {449} (Axial) (mg $l^{-1}$) | **Cr** 267.716 {126} (Axial) (mg $l^{-1}$) | **Cu** 327.396 {103} (Axial) (mg $l^{-1}$) | **Fe** 259.940 {130} (Radial) (mg $l^{-1}$) | **Mg** 280.270 {120} (Radial) (mg $l^{-1}$) |
| DL | 0.0002 | 0.0007 | 0.0060 | 0.0184 | 0.0029 |
| A-1 | n.d. | 0.00 | 0.01 | 0.08 | 43.17 |
| A-2 | n.d. | 0.00 | 0.02 | n.d. | 44.49 |
| A-3 | n.d. | 0.00 | 0.01 | 0.09 | 45.64 |
| B-1 | n.d. | 0.00 | 0.01 | 0.05 | 43.85 |
| B-2 | n.d. | 0.00 | 0.03 | 0.10 | 43.99 |
| B-3 | n.d. | 0.00 | 0.02 | n.d. | 46.40 |
| C-1 | n.d. | n.d. | n.d. | n.d. | 40.67 |
| C-2 | n.d. | n.d. | n.d. | 0.18 | 42.06 |
| C-3 | n.d. | n.d. | n.d. | 0.12 | 40.00 |
| Limit values | 0.0050 | 0.05 | 1.00 | 0.20 | 10.00 |
| Sample | **Mn** 259.373 {130} (Axial) (mg $l^{-1}$) | **Na** 589.592 {57} (Radial) (mg $l^{-1}$) | **Ni** 221.647 {452} (Axial) (mg $l^{-1}$) | **Pb** 220.353 {453} (Axial) (mg $l^{-1}$) | **Sb** 231.147 {446} (Axial) (mg $l^{-1}$) |
| DL | 0.0001 | 0.0258 | 0.0004 | 0.0015 | 0.0082 |
| A-1 | 0.02 | 34.94 | 0.00 | n.d. | n.d. |
| A-2 | 0.05 | 34.12 | 0.01 | n.d. | n.d. |
| A-3 | 0.05 | 34.04 | 0.00 | n.d. | n.d. |
| B-1 | 0.01 | 31.13 | 0.00 | 0.00 | n.d. |
| B-2 | 0.02 | 30.69 | 0.00 | 0.00 | n.d. |
| B-3 | 0.03 | 31.96 | 0.00 | n.d. | n.d. |
| C-1 | 0.01 | 32.72 | 0.00 | n.d. | n.d. |
| C-2 | 0.00 | 34.69 | n.d. | 0.00 | n.d. |
| C-3 | 0.00 | 33.06 | 0.00 | 0.00 | n.d. |
| Limit values | 0.05 | 200.00 | 0.02 | 0.01 | 0.0050 |

Explanatory notes: DL – detection limit (Vyhláška č. 252/2004 Sb, 2004), n.d. – under detection limit.

The concentration values of elements were again considered at the outflow from the sedimentation tanks, ie. A-3, B-3, and C-3. In this case, the only elements which do not meet the limit values for drinking water are calcium and magnesium. The increased levels of these substances serve to change the taste of the water and increase its hardness. Since these substances are not toxic in nature and have no significant effect on the water being used for rinsing vegetables, their removal from the wastewater is not necessary. Table 5 shows the concentrations of selected inorganic anions in wastewater. As a comparison, the limit values for drinking water are used (Vyhláška č. 252/2004 Sb, 2004).

**Table 5.** Concentrations of selected inorganic anions in wastewater

| Sample | **$F^-$** (mg $l^{-1}$) | **$Cl^-$** (mg $l^{-1}$) | **$NO_2^-$** (mg $l^{-1}$) | **$NO_3^-$** (mg $l^{-1}$) | **$SO_4^{2-}$** (mg $l^{-1}$) | **pH** ( - ) | **Conductivity** (µS $cm^{-1}$) |
|---|---|---|---|---|---|---|---|
| DL | 0.0005 | 0.0012 | 0.0023 | 0.0042 | 0.0031 | | |
| A-1 | 0.49 | 73.28 | 0.68 | 109.73 | 244.03 | 7.76 | 1353 |
| A-2 | 0.46 | 78.26 | 32.55 | 57.47 | 251.36 | 7.37 | 1375 |
| A-3 | 0.69 | 77.10 | 22.01 | 54.58 | 242.87 | 7.13 | 1382 |
| B-1 | 0.54 | 75.18 | 5.22 | 44.06 | 249.93 | 7.78 | 1379 |
| B-2 | 0.53 | 73.24 | 3.45 | 6.32 | 241.67 | 7.97 | 1361 |
| B-3 | 0.51 | 73.75 | 6.94 | 9.01 | 244.69 | 7.73 | 1414 |
| C-1 | 0.52 | 84.62 | 14.07 | 74.36 | 255.27 | 7.35 | 1395 |
| C-2 | 0.67 | 79.82 | 0.03 | 0.31 | 238.19 | 7.37 | 1383 |
| C-3 | 0.86 | 80.85 | 35.60 | 7.68 | 244.18 | 7.57 | 1393 |
| Limit values | 1.50 | 100.00 | 50.00 | 50.00 | 250.00 | 6.5-9.5 | 1250 |

Explanatory notes: DL – detection limit, Limit values for drinking water is used from Vyhláška č. 252/2004 Sb, 2004.

An increased concentration of nitrates was detected through an analysis of inorganic anions, and a greater conductivity value was determined through measurements. An increased conductivity value indirectly indicates that the contents of mineral substances to be found in the water are closely related to the higher volumes of calcium and magnesium. These increased values have a negative impact on the drinking water that is used; however, for the purposes of the post-harvest treatment of vegetables, these exceeded limit values are acceptable.

## CONCLUSION

Sedimentation tanks reduced the content of substances in wastewater, in particular undissolved substances. However, for the reuse of this water in the line for the post-harvest treatment of vegetables, it would be suitable to include another level of wastewater purification (Council Directive 98/83/EC). The concentrations of some elements on the outlet from sedimentation tanks increased slightly. The limit values are also exceeded for nitrates and conductivity.

On the basis of the values measured, an additional water purification step was recommended so that the water can be reused. This was mainly a reduction in undissolved substances. In order to select a suitable device, the highest TS value was overestimated to 0.7g $l^{-1}$. A gravitational flow-through drum filter was recommended, which will be placed on the outlet from the sedimentation tanks. In this case, the water would be exchanged once a week for new water taken from the water pipe line.

ACKNOWLEDGEMENTS. This work was financially supported by the Czech University of Life Sciences Prague (Internal Project No CIGA 20162016).

## REFERENCES

Alsiņa, I., Dūma, M., Dubova, L., Šenberga, A. & Daģis, S. 2016. Comparison of different chlorophylls determination methods for leafy vegetables. *Agronomy Research* **14**(2), 309–316.

Ash, C., Drabek, O., Tejnecky, V., Jehlicka, J., Michon, N. & Boruvka, L. 2016. Sustainable Soil Washing: Shredded Card Filtration of Potentially Toxic Elements after Leaching from Soil Using Organic Acid Solutions. *Plos one* **11**(2). Article number: e0149882. Accession Number: WOS:000371276100173. ISSN 1932-6203.

Borys, N. & Küüt, A. 2016. The influence of basic soil tillage methods and weather conditions on the yield of spring barley in forest-steppe conditions. *Agronomy Research* **14**(2), 317–326.

Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption.

Dubrovskis, V. & Plume, I. 2015. Anaerobic digestion of vegetables processing wastes with catalyst metaferm. *Agronomy Research* **13**(2), 294–302.

Horáková, M. 2003. *Water analysis. 2nd edition*. VŠCHT 2003, 256 pp. (in Czech).

Goliáš, J. 2014. *Storage and processing of fruits and vegetables*. *1st edition*. MEDELUB 2014, 281 pp. (in Czech).

Kern, J., Reimann, W. & Schlüter, O. 2006. Treatment of recycled carrot washing water. *Environmental Technology* **27**(4), 459–466.

Mercl, F., Tejnecky, V., Szakova, J. & Tlustos, P. 2016. Nutrient dynamics in soil solution and wheat response after biomass ash amendments. *Agronomy journal* **108**(6), 2222–2234.

Nařízení vlády č. 401/2015. *Government regulation on indicators values of permissible pollution of surface water treatment plants, check the details of the permit to discharge wastewater to make surface waters and sewers and for sensitive areas.* Czech law. (in Czech).

Pao, S., Long, W., Kim, C. & Kelsey, D.F. 2012. *Produce Washers. Decontamination of Fresh and Minimally Processed Produce. 2nd edition*. Wiley-Blackwell, pp. 87–103.

Simons, L.K. & Sanquansri, P. 1997. Advances in the washing of minimally processed vegetables. *Food Australia* **49**(2), 75–80.

Vaculík, P., Maloun, J., Chládek, L. & Přikryl, M. 2013. Disintegration process in disc crushers. *Research in Agricultural Engineering* **59**(3), 98–104.

Vyhláška č. 252/2004 Sb. *Decree laying down hygienic requirements for drinking hot water frequency and scope of drinking water control.* Czech law. (in Czech).

## INSTRUCTIONS TO AUTHORS

Papers must be in English (British spelling). English will be revised by a proofreader, but authors are strongly urged to have their manuscripts reviewed linguistically prior to submission. Contributions should be sent electronically. Papers are considered by referees before acceptance. The manuscript should follow the instructions below.

**Structure:** Title, Authors (initials & surname; an asterisk indicates the corresponding author), Authors' affiliation with postal address (each on a separate line) and e-mail of the corresponding author, Abstract (up to 250 words), Key words (not repeating words in the title), Introduction, Materials and methods, Results and discussion, Conclusions, Acknowledgements (optional), References.

**Layout, page size and font**

- Use preferably the latest version of **Microsoft Word**, doc., docx. format.
- Set page size to **B5 Envelope or ISO B5 (17.6 x 25 cm)**, all margins at 2 cm.
- Use single line spacing and justify the text. Do not use page numbering. Use indent 0.8 cm (do not use tab or spaces instead).
- Use font Times New Roman, point size for the title of article **14 (Bold)**, author's names 12, core text 11; Abstract, Key words, Acknowledgements, References, tables and figure captions 10.
- Use *italics* for Latin biological names, mathematical variables and statistical terms.
- Use single ('…') instead of double quotation marks ("…").

**Tables**

- All tables must be referred to in the text (Table 1; Tables 1, 3; Tables 2–3).
- Use font Times New Roman, regular, 10 pt. Insert tables by Word's 'Insert' menu.
- Do not use vertical lines as dividers; only horizontal lines (1/2 pt) are allowed. Primary column and row headings should start with an initial capital.

**Figures**

- All figures must be referred to in the text (Fig. 1; Fig. 1 A; Figs 1, 3; Figs 1–3). Use only black and white or greyscale for figures. Avoid 3D charts, background shading, gridlines and excessive symbols. Use font **Arial** within the figures. Make sure that thickness of the lines is greater than 0.3 pt.
- Do not put caption in the frame of the figure.
- The preferred graphic format is EPS; for half-tones please use TIFF. MS Office files are also acceptable. Please include these files in your submission.
- Check and double-check spelling in figures and graphs. Proof-readers may not be able to change mistakes in a different program.

**References**

- **Within the text**

In case of two authors, use '&', if more than two authors, provide first author 'et al':

Smith & Jones (1996); (Smith & Jones, 1996);
Brown et al. (1997); (Brown et al., 1997)

When referring to more than one publication, arrange them by following keys: 1. year of publication (ascending), 2. alphabetical order for the same year of publication:

(Smith & Jones, 1996; Brown et al., 1997; Adams, 1998; Smith, 1998)

- **For whole books**

Name(s) and initials of the author(s). Year of publication. *Title of the book* (*in italics*). Publisher, place of publication, number of pages.

Shiyatov, S.G. 1986. *Dendrochronology of the upper timberline in the Urals*. Nauka, Moscow, 350 pp. (in Russian).

- **For articles in a journal**

Name(s) and initials of the author(s). Year of publication. Title of the article. *Abbreviated journal title* (*in italic*) volume (in bold), page numbers.

Titles of papers published in languages other than English, German, French, Italian, Spanish, and Portuguese should be replaced by an English translation, with an explanatory note at the end, e.g., (in Russian, English abstr.).

Karube, I. & Tamiyra, M.Y. 1987. Biosensors for environmental control. *Pure Appl. Chem.* **59**, 545–554.

Frey, R. 1958. Zur Kenntnis der Diptera brachycera p.p. der Kapverdischen Inseln. *Commentat.Biol.* **18**(4), 1–61.

Danielyan, S.G. & Nabaldiyan, K.M. 1971. The causal agents of meloids in bees. *Veterinariya* **8**, 64–65 (in Russian).

- **For articles in collections:**

Name(s) and initials of the author(s). Year of publication. Title of the article. Name(s) and initials of the editor(s) (preceded by In:) *Title of the collection (in italics)*, publisher, place of publication, page numbers.

Yurtsev, B.A., Tolmachev, A.I. & Rebristaya, O.V. 1978. The floristic delimitation and subdivisions of the Arctic. In: Yurtsev, B. A. (ed.) *The Arctic Floristic Region*. Nauka, Leningrad, pp. 9–104 (in Russian).

- **For conference proceedings:**

Name(s) and initials of the author(s). Year of publication. Name(s) and initials of the editor(s) (preceded by In:) *Proceedings name (in italics)*, publisher, place of publishing, page numbers.

Ritchie, M.E. & Olff, H. 1999. Herbivore diversity and plant dynamics: compensatory and additive effects. In: Olff, H., Brown, V.K. & Drent R.H. (eds) *Herbivores between plants and predators. Proc. Int. Conf. The 38th Symposium of the British Ecological Society*, Blackwell Science, Oxford, UK, pp. 175–204.

.....……

**Please note**

- Use '.' (not ',') for decimal point: 0.6 ± 0.2; Use ',' for thousands – 1,230.4;
- Use '–' (not '-') and without space: pp. 27–36, 1998–2000, 4–6 min, 3–5 kg
- With spaces: 5 h, 5 kg, 5 m, 5°C, C : D = 0.6 ± 0.2; $p < 0.001$
- Without space: 55°, 5% (not 55 °, 5 %)
- Use 'kg $ha^{-1}$' (not 'kg/ha');
- Use degree sign ' ° ' : 5 °C (not 5 $^{O}$ C).