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# FOOD SCIENCE and TECHNOLOGY

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# Differences in cadmium accumulation and induced changes in root anatomical structures in plants used for food

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**Abstract.** A rapid urbanization passes all over the world thus the effect of chemicals, including heavy metals, increases on plants. Heavy metal pollution poses a serious hazard to humans' health, and it uptake into plants is the primary way through which it can enter the food chain. The goal of this study was to investigate the impact of cadmium (Cd) contamination on plant growth responces, Cd uptake, and changes in the root anatomical structures as species-specific reaction to Cd stress. The vegetation experiment was carried out with monocotyledon *Hordeum vulgare* L. and dicotyledonous *Lactuca sativa* L. The plants were grown in quartz sand under controlled optimal growth conditions. Changes in the root structure and Cd accumulation were studied at five levels of Cd added as $Cd(NO_3)_2$ 4 $H_2O$ solution in substrate. The level of Cd in the air-dry plant material was estimated by an atomic absorption spectrometer. To identify structural changes in the plant roots which were caused by Cd accumulation cross sections were cut using microtome and stained with Astra Blue/Safranin for observations using a light microscope. Barley and lettuce growth and development were significantly influenced by increasing the amount of Cd in substrate. There were differences in the ability to accumulate Cd in above-ground plant parts depending on a model object. Substrate contamination with Cd caused significant changes in the root anatomical structures. The obtained results confirmed significance of anatomical and physiological studies to reveal species-specific plant response to Cd stress to avoid heavy metal entrance in the food.

**Key words:** barley, lettuce, cadmium uptake, root anatomy, growth responses.

## INTRODUCTION

Soil contamination with heavy metals has occurred over thousands of years. Soil can be contaminated as the result of natural processes or human activity (Meuser & Van der Graaff, 2011; Swartjes, 2011). European Environmental Agency has found out that there are 2.5 million potentially contaminated sites in Europe and about 14% of them are expected as contaminated. The main types of local sources of contamination in Europe are waste disposal (municipal and industrial waste disposal), industrial and commercial activities (mining, oil extraction and production, power plants), military activities (military sites, war affected zones), storages (oil storage, obsolete chemicals storage, other storages), transport spills on land (sites with spills of oil and other hazardous substances), nuclear (nuclear operations) and other sources (van Liedekerke et al., 2014).

In Europe the main contaminant groups are heavy metals (34.8%) and mineral oils (23.8%) (van Liedekerke et al., 2014).

Among phytotoxic heavy metals, Cd is one of considerable importance due to high water solubility, mobility, and toxicity even in low levels. Severe toxicity of Cd is based on mutagenic and carcinogenic features and high accumulation capacity in plants tissues (Siedlecka, 1995; di Toppi & Gabbrielli, 1999; Lux et al., 2011b). Thus Cd has become one of the most harmful pollutants in agricultural soils with high potential to enter the food chain (Peralta-Videa et al., 2009; McLaughlin et al., 2011) and cause a negative effect on humans' health (Swartjes & Cornelis, 2011). In general, plants have different ability to accumulate heavy metals from soil - vegetable species can be listed in increasing order of Cd accumulation: French bean, beetroot, radish, pea, carrot, and broccoli < potato, tomato, zucchini, and sweetcorn < onion, leek, parsnip < turnip < cabbage, kale < lettuce, spinach (McLaughlin et al., 2011).

The interaction of heavy metals with various biochemical and physiological processes in plants is widely studied (Hall, 2002; Clemens, 2006), however the general overview of processes, taking place in heavy metal-affected cells, are only partially understood. Martin et al. (2006) have revealed that heavy metals reduce shoot: root biomass ratio in vegetables. It is well documented that heavy metals compete with nutrients, causing mineral disturbance in plants, and as a result their growth becomes slower (Siedlecka, 1995; Vollenweider et al., 2006; Ghnaya et al., 2007; Titov et al., 2007). Visual symptoms of Cd phytotoxicity as leaf necrosis, browning of roots, reducting of root diameter and branching, increasing fragility have been found for *Pisum sativum, Lactuca sativa, Spinacia oleracea* (Fusconi et al., 2006; Osvalde & Paegle, 2007).

There are two kinds of heavy metals which are referred to micronutrients essential for plant metabolism (Fe, Cu, Zn, Mn and Mo) and not essential, such as Cd, Hg and Pb (Marschner, 1995; Siedlecka, 1995). Both can cause toxicities when they are present in excess. In spite of the different mobility of metals in soil and plants, the root system, in general, accumulates significantly higher concentrations of heavy metals (Abe et al., 2008) and it is one of the main targets of the toxic effects. Consequently, the disturbed root metabolism directly or indirectly affects other physiological processes in plants (Titov et al., 2007).

During the last years particular scientific attention was paid on phytoremediation techniques of contaminated soils as well as on plants with exceptional metal-accumulating capacity – hyperaccumulators (Raskin & Ensley, 2000). In general, metal hyperaccumulating species have been identified at least in 45 plant families and new species or populations continue to be discovered (Kraemer, 2003). Economically important crop species including barley (*Hordeum vulgare*) recently have been identified as plants for efficient uptake and accumulation of heavy metals with a phytoremediation potential (Vassilev et al., 1998; Sękara et al., 2005; Nadgórska-Socha et al., 2013).

The physiological mechanisms and main strategies of different species related to the metal accumulation, compartmentalization, and detoxification have still not been investigated in detail. Many studies have revealed the changes in the morphological traits of plants and internal structures of roots as epidermis, cortex parenchyma, exodermis, vascular cynlinder, sclerenchyma, Casparian strips, and the xylem pipes under heavy metal stress in the range of plants (Hose et al., 2001; Enstone et al., 2003; Probst et al.,

2009). These changes, occurring in plants, can be species and tissue specific (Hermle et al., 2006; Lux et al., 2011a).

Although, response of plants to Cd treatment is intensively studied there is still limited knowledge concerning the particular role of root anatomical structure of monocotyledons and dicotyledons in fixing and transport of metal ions. Cd can cause changes in the root morphology and anatomy of plants. There are mechanisms – physiological, anatomical and physical - how plants prevent Cd uptake in the root and transport to xylem (Lux et al., 2011a; Lux et al., 2011b). Some of them are: production of Cd-chelates, accelerated development of endodermis and exodermis (Hose et al., 2001; Enstone et al., 2003; Lux et al., 2011a), formation of hypodermal periderm (Lux et al., 2011b) and peri-endodermal layer of cells with lignified cell walls (Zelko et al., 2008). The increased thickness of the abaxial and adaxial sclerenchyma and pericycle tissues in *Brachiaria decumbens*, caused by heavy metals, could be related to adsorption of metals in the cell walls, constituting an alternative pathway for allocation of these ions and preventing their translocation to photosynthetic tissues (Gomes et al., 2011).

Another changes in the root anatomical structures caused by presence of heavy metals were observed by Vollenweider et al. (2006). Roots of *Salix viminalis* cultivated in the presence of Cd formed thickened cell walls of the collenchymas and pericycle, with higher concentrations of metal than the other tissues.

It is known from previous studies that monocotyledons and dicotyledons can accumulate different Cd and lead (Pb) amount from soil (Martin et. al., 2006; Osvalde & Paegle, 2007; Abe et al., 2008; Chang et al., 2014). As morphologically differentiated inner cell layer of cortex - root endodermis has an important effect on the transport of soil solution between the root cortex and vascular cylinder, endodermis typical for monocotyledons with its Casparian stripes acts as an important barrier that holds up heavy metals (Enstone et al., 2003; Gomes et al., 2011).

A morphologically differentiated inner cell layer of cortex - endodermis typically surrounds the pericycle (Evert, 2006). In the roots, in which the secondary growth is not present, endodermis has several steps of differentiation. The first is formation of thickened and lignified Casparian strips in anticlinal walls of endodermis (Evert, 2006). During the second step the cell wall covers with a thin suberin film to an inner side of cell thus separating the Casparian strips from cytoplasm. The third step characterizes by an irregular thickening of endodermis cell wall. If the thickening occurs in all the cell wall except the outer one, the U – like endodermis forms. Most of monocots and just some dicots have the third step of endodermis differentiation (Evert, 2006).

Since a lot of field crops are monocots with endodermis consisting of U – like form cells, endodermis may have a relevant role in preventing a free penetration of pollutants through the apoplast.

Therefore, knowledge on modifications in the structure of root tissues could provide insights on differences in the root system functioning and capability of heavy metal accumulation among the plant species. The goal of this study was to investigate the impact of Cd contamination on plant growth responses, Cd uptake, and changes in the root anatomical structures as species specific reaction to Cd stress.

## MATERIALS AND METHODS

The vegetation experiment was carried out with a monocotyledon barley (*Hordeum vulgare* L., cv. 'Ansis') and a dicotyledonous lettuce (*Lactuca sativa* L., cv. 'Grand Rapid') as model plants. These plants were grown up in 1 L polyethylene containers from seeds (18 barley plants per pot and three lettuce plants per pot) under controlled growth conditions – day/night temperature + 20/18 °C, photoperiod light/dark 16/8 h, humidity of substrate 60–65%, photon flux density 160 μmol $m^{-2}$ $s^{-1}$ supplied by fluorescent tubes). Quartz sand was used as substrate. Humidity of the substrate was maintained throughout the experiment gravimetrically using deionized water.

Five levels of Cd (added as $Cd(NO_3)_2 \cdot 4\ H_2O$ solution): 0, 3, 6, 9, 12 mg $L^{-1}$ for barley and 0, 1, 2, 4, 6 mg $L^{-1}$ for lettuce were added in substrate. Nutrient solution, containing optimal concentrations of macroelements and microelements (in mg $L^{-1}$: N 120, P 60, K 150, Ca 800, Mg 50, S 60, Mn 1.5, Zn 1, Cu 0.5, Mo 0.02, B 0.2, Fe 30) (Osvalde, 2011), was added in substrate for all treatments. All the nutrients were provided with complete nutrient solution, Ca as $CaCO_3$ in each pot at the beginning of the experiment.

The vegetation experiment ran for 43 days. Plants were collected on the day of $16^{th}$, $23^{rd}$, $29^{th}$, $36^{th}$ and $43^{rd}$ of the experiment. Biomass of plans was estimated during the experiment. Roots were separated from shoots and washed in distilled water. Fresh weight of leaves and roots was determined for one plant.

Plant material was dried at 64 °C to a constant weight. Plant material was dry mineralized with $HNO_3$ vapours and dissolved in HCl (Rinkis et al., 1987). The level of Cd in air-dry plant material was estimated by an atomic absorption spectrometer Perkin Elmer AAnalyst 700A, acetylene-air flame. For control treatment Cd analyses were conducted on a graphite furnace equipped atomic absorption spectrometer Perkin Elmer AAnalyst 700 (Anonymous, 2000).

To identify structural changes in the plant roots, caused by Cd accumulation, 15 μm cross sections were cut using Leica SM 2010R microtome and stained with Astra Blue/Safranin. Cross sections were made in two regions – at the distance of 10 mm from a root apex and 5 mm from a root base. Investigation and photographs of micro slides were made using digital microscope Leica DM5500 equipped with a digital camera Leica DFC490. Images were analysed using graphic workstation Dell Precision T7400 and software Image-Pro Plus 6.2.

The statistical analysis of results was done using MS Excel 2013. Standard errors (SE) were calculated in order to reflect the mean of the results. The Student's *t*-test 'Two-Sample Assuming Equal Variances' ($p < 0.05$) was used for testing the differences between the treatments.

## RESULTS AND DISCUSSION

Heavy metal pollution in soil had different impact on the process of biomass production of barley and lettuce. At control conditions Cd0 barley biomass gradually raised up to the fifth leaf stage, at Cd3 – to the sixth leaf stage, at Cd6, Cd9 and C12 – to the fourth leaf stage, while lettuce biomass of control treatment Cd0 gradually raised up to the ninth leaf stage, at Cd1 and Cd2 – to the eighth leaf stage and Cd4 and Cd6 – to the sixth leaf stage.

In general, plant exposure to Cd treatments resulted in decreasing fresh biomass of plant leaves and roots significantly. At the highest pollution level (Cd12) biomass of barley leaves was 55.45% and of roots 36.79% from the control (Cd0) at the end of the experiment (Fig. 1, A, B). There was no significant impact on biomass of barley leaves and roots at the low pollution level of Cd 3 mg $L^{-1}$ in the substrate on the 29$^{th}$ day (*t*-test, $p < 0.05$).

In plant fresh weight a considerable decrease was also found for lettuce (Fig. 2, A, B). At the end of the experiment, Cd6 treatment resulted in massive inhibition of leaf and root biomass (29.50% and 21.76%, respectively, of the control level).

It is notable that the highest root biomass was found for low Cd concentrations in substrate (for barley Cd3, for lettuce Cd1 and Cd2). For lettuce this phenomenon was typical at the beginning of ontogenesis.

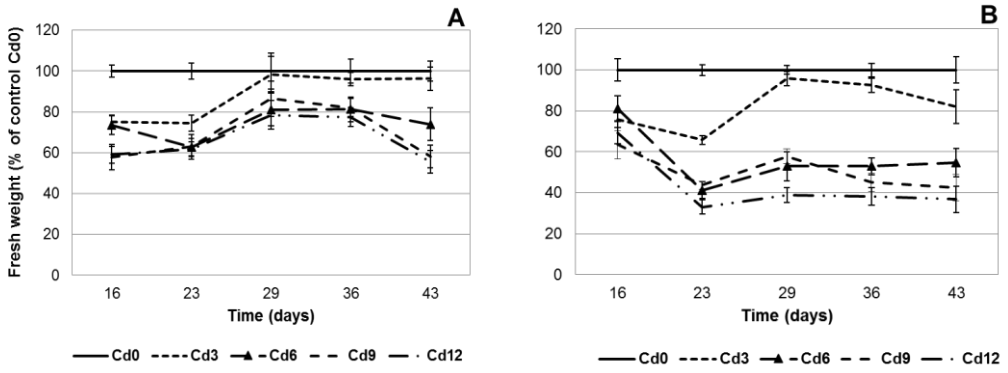


**Figure 1.** Fresh weight (% of control) of the leaves (A) and roots (B) of *H. vulgare* L. at five levels of Cd added in substrate, ± SE.

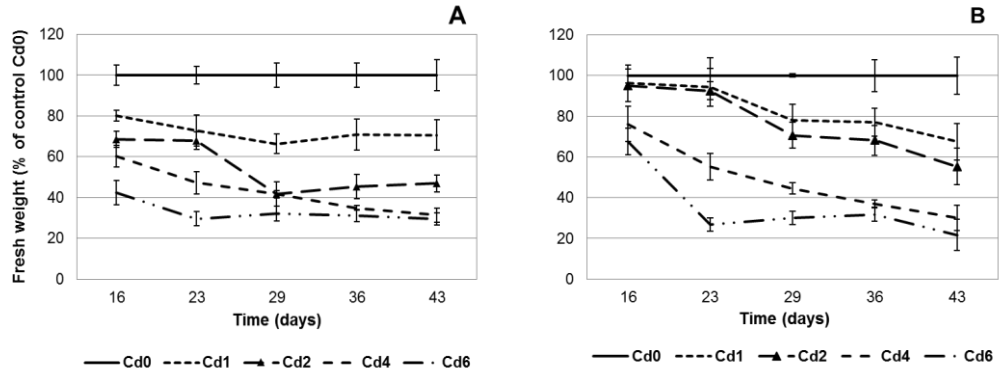


**Figure 2.** Fresh weight (% of control) of the leaves (A) and roots (B) of *L. sativa* L. at five levels of Cd added in substrate, ± SE.

Similar tendencies of changes in plant biomass of leaves and roots under Cd pollution were confirmed by other studies which found that low concentrations of Cd could contribute to the activation of physiological processes in the plant, while at high concentrations there was high possibility of irreversible, even structural changes (Hart et al., 1998; Wójcik & Tukiendorf, 1999, Titov et al., 2007). In our study the results revealed that significant changes were typical for barley at Cd pollution level from 6 to 12 mg $L^{-1}$ in substrate, for lettuce from 2 to 6 mg $L^{-1}$ in substrate.

During the recent years increasing experimental evidence has been associated to Cd toxicity with antagonistic reactions of Cd and essential nutrients (N, P, K, Ca, Mg, Fe, Cu, B, Mn and Zn), alteration of membrane permeability, generation of oxidative stress. Thus, Cd toxicity may result from: a) disturbances in Zn uptake and transport, due to similar pathways; b) replacing of K and inactivation of stomata; c) inactivation of photosynthesis (Cd inactivates water photolysis, reaction-centers of photosystems, electron transport, carboxylation enzyme *Rubisco* and phosphoglycerol synthetase activity) etc. (Siedlecka & Krupa, 1996; Grant et al., 1998; Arazi et al., 1999; di Toppi & Gabbrielli, 1999; Siedlecka & Krupa, 1999; Clemens, 2001; Šeršeň & Královά, 2001). The plant vitality and biomass reduced in the result of inactivation of these physiological processes. Barceló et al. (1990) and Sandalio et al. (2001) have mentioned that reduction of plant biomass has been the result of nutrition and water unbalance in plants. During the experiment, it was observed that both barley and lettuce roots changed their colour (they became darker yellow) and became thinner and more fragile. As well as there were visual changes in leaves as chlorotic spots observed and at the end of the experiment leaves became necrotic.

Plant exposure in increasing levels of Cd resulted in a progressive inhibition of growth and simultaneous accumulation of Cd both in leaves and roots of plants. The highest concentrations of Cd were established for both barley and lettuce roots and leaves (Figs 3, 4) at the beginning of ontogenesis (from the $16^{th}$ to $23^{rd}$ day of the experiment).

Although, Cd concentrations in the roots were much higher than in leaves of studied plants, lettuce and barley exhibited specific differences in the ability to accumulate Cd in leaves. In general, Cd accumulation occurred more intensively in lettuce leaves, compared to barley. Thus, on the $29^{th}$ day of the experiment under a 6 mg $L^{-1}$ level of Cd in the substrate (Cd6), the concentration of Cd in lettuce leaves reached 56.0 mg $kg^{-1}$ while in barley leaves 25.4 mg $kg^{-1}$ (Figs 3, A; 4, A). More seriously affected leaf biomass of lettuce was also found in these conditions: fresh weight of lettuce aboveground parts was 2.5 times lower than for barley and reached only 30% of control level. Our data support previous findings that leafy vegetables can accumulate the highest value of heavy metals (Pandey & Pandey, 2009; Ngole, 2011; Liu et al., 2012; Chang et al., 2014).

The obtained results confirmed that metal accumulation mainly depends on plant species. Although, Cd is considered to be one of the most mobile heavy metals with high translocation possibilities to plant above-ground tissues (Lux et al., 2011a; (Vassilev et al., 2004; Sękara et al., 2005;), it mainly accumulates in the plant roots. At the end of the experiment Cd concentration in barley roots under treatments, respectively, Cd3, Cd6, Cd9, Cd12 was 12.7, 16.6, 11.8, and 13.9 times higher than in the leaves (Fig. 3, B). These ratios for lettuce were significantly lower - at the end of the experiment Cd level of lettuce roots under treatment Cd1 was 3.2 times higher than in the leaves, Cd2 – 3.8, Cd4 – 5.2 and Cd6 – 6.0 (Fig. 4, B). As reduced translocation of heavy metals to the

plant shoots a possible mechanism of metal tolerance appears (Lux et al., 2011a), the obtained results confirmed that barley roots act as a physiologically active protection barrier restricting root-shoot translocation of Cd.

Uptake and transport processes have been recognized as the central mechanism of metal detoxification and tolerance in plants (di Toppi & Gabbrielli, 1999; Hall, 2002; Enstone et al., 2003; Sękara et al., 2005; Clemens, 2006; Gomes et al., 2011; Lux et al., 2011a; Lux et al., 2011b; Vaculík et al., 2012). Therefore, the investigations were done to reveal the particular role of the root anatomical structure in fixing and transport of Cd to provide more insights in differences of monocotyledons and dicotyledons.

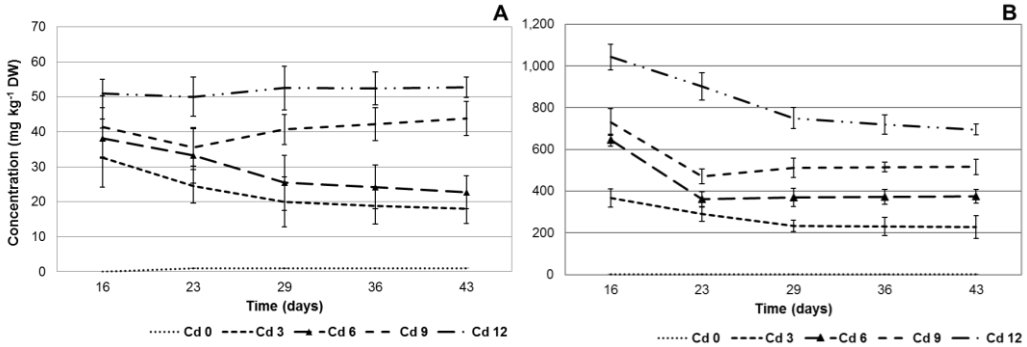


**Figure 3.** Cadmium concentrations (mg $kg^{-1}$ DW) in the leaves (A) and roots (B) of *H. vulgare* L. at five levels of Cd added in substrate, ± SE.

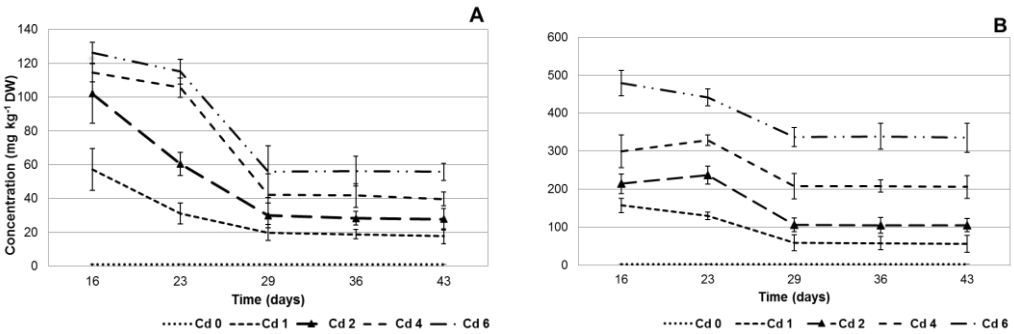


**Figure 4.** Cadmium concentrations (mg $kg^{-1}$ DW) in the leaves (A) and roots (B) of *L. sativa* L. at five levels of Cd added in substrate, ± SE.

Current study suggests that at the beginning of ontogenesis Cd transport from the roots to the leaves in the barley (monocotyledons) can be delayed by Casparian stripes in endoderm, later by the cell wall thickening of endoderm and pericycle cells (Fig. 5). At the beginning of ontogenesis the cell wall thickening was less expressed in lettuce roots (Fig. 5, B). The differences in development of endodermis under equal level of Cd contamination (Cd6) were found – while endodermis of barley formed lignified anticlinal and inner periclinal U-shape cell walls as well as peri-endodermis (Fig. 5, A), only Casparian bands in the root endodermis cells of lettuce plants were present and formation of peri-endodermis absent (Fig. 5, B).

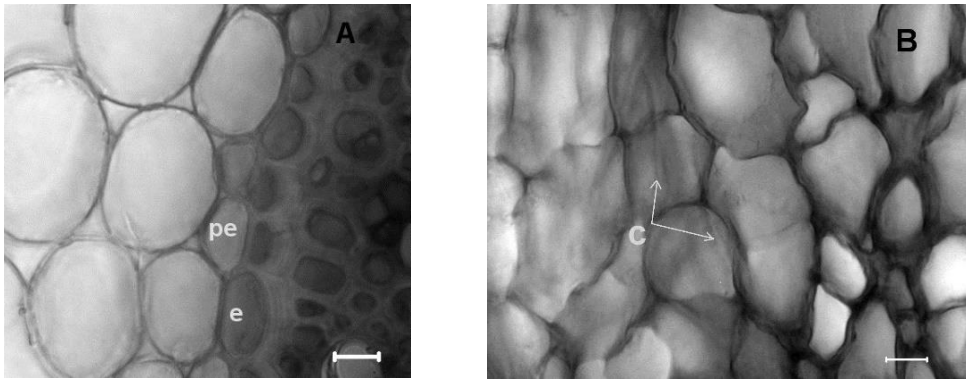


**Figure 5.** Cross section of *H. vulgare* L. root base (A) and cross section of *L. sativa* L. main root base (B) exposed to Cd6 and on the 29th day of the experiment. Abbreviations: e – endodermis; pe – peri-endodermis; c – Casparian bands. Scale bar: 5 µm (A), 20 µm (B).

Our study has shown that a peri-endodermal layer of cells with lignified cell walls was present in the base of barley root starting from the 23rd day of the experiment (Fig. 6). Although, some authors report that these apoplasmic barriers develop closer to the root apex when roots are exposed to high concentrations of potentially toxic elements (Zelko et al., 2008; Lux et al., 2011a). The impact of Cd contamination level Cd3 on the formation of peri-endodermis can be observed starting from the 43rd day of the experiment when 3 cells were found. The number of peri-endoderm cells increases depending on Cd treatment level. Most relevant differences in number of peri-endoderm cells were stated from the 43rd day of the experiment – while they were absent in the control plants, in Cd3, Cd6, Cd9 and Cd12 treatments 3, 6, 11 and 15 cells were found respectively (Fig. 6). Formation of peri-endodermis in lettuce roots was not observed.

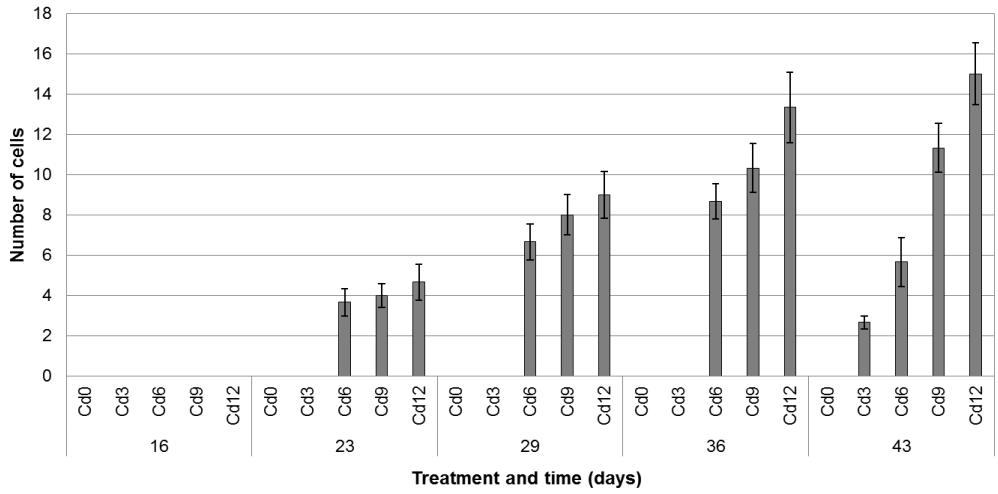


**Figure 6.** Number of peri-endodermis cells in *H. vulgare* L. root base exposed to five levels of Cd added in substrate, ± SE.

The studies on changes of the root anatomical structure in conditions of Cd toxicity reveal the presence of lignified pericycle cell walls and the lack of passage cells in endodermis of barley under Cd6 (Fig. 7, A), while under Cd0 passage cells are present and proximal pericycle cell walls remain unlignified and maintained parenchymatic character (Fig. 7, B). This available evidence suggests that passage cells of endodermis allow the passing solutions towards vascular cylinder by symplast to play an active role in ion uptake and could be important for Cd uptake and transfer (Peterson & Enstone, 1996). Penetrating the soil solution is ensured by the transpiration in tracheary elements of the radial vascular bundle of root, the passage cells in their turn are located opposite to xylem elements and ensure the penetration in vascular cylinder only via the symplast (Gomes et al., 2011).

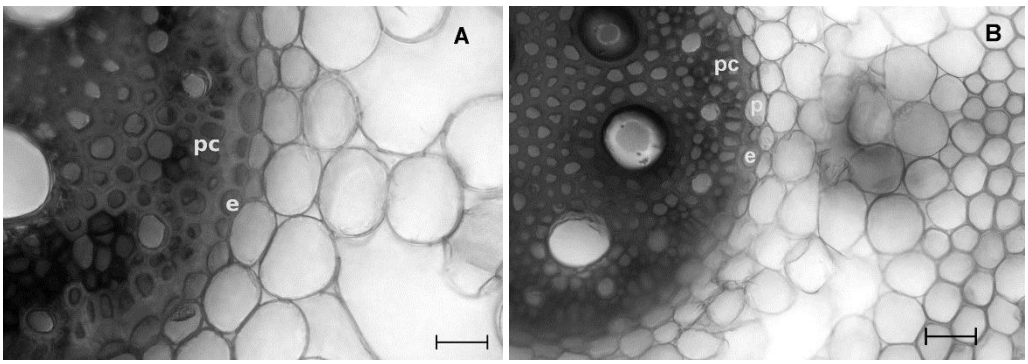


**Figure 7.** Cross section of *H. vulgare* L. root base exposed to Cd6 (A) and Cd0 (B) on the 29$^{th}$ day of the experiment. Abbreviations: e – endodermis; pc – pericycle; p – passage cells. Scale bar: 30 μm (A), 50 μm (B).

According to Nagahashi et al. (1974) the effectiveness of the Casparian strips as a barrier to apoplastic movement has been demonstrated at the electron microscope level by the use of maize roots. Thus, heavy metals cannot penetrate the endodermis to enter the stele through apoplast – it is allowed only by symplastic structures of passage cells (Enstone et al., 2003; Gomes et al., 2011).

## CONCLUSIONS

This study suggests that Cd accumulation in monocotyledon and dicotyledon plant leaves and roots differs depending on changes in the root anatomical structures caused by Cd pollution. Barley and lettuce exposure to Cd treatments resulted in decreasing fresh biomass of leaves and roots significantly. Significant changes were characteristic for barley at Cd pollution level from 6 to 12 mg $L^{-1}$ in substrate, for lettuce from 2 to 6 mg $L^{-1}$ in substrate. The obtained results confirm that metal accumulation highly depends on plant species: lettuce and barley exhibit specific differences in the ability to accumulate Cd in their leaves. Changes in anatomical structures of barley roots – endoderm cell walls thickening and lignification, lack of passage cells in endodermis, pericycle cell walls thickening as well as formation of peri-endodermis – act as an active

protection barrier restricting root-shoot translocation of cadmium. At the beginning of ontogenesis the corresponding responses were less expressed in lettuce roots.

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# The influence of microelements selenium and copper on the rye malt amylase activity and flour technological properties

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**Abstract.** The positive effect of microelement selenium (Se) on the amylase activity and technological properties of malt is well known. Copper (Cu) is an essential microelement required for the normal functioning of living organisms, plants and most microorganisms. The aim of the current research was to investigate the interaction of two microelements - copper and selenium and its influence on the rye malt and flour properties. Rye grain of 96% viability were soaked and germinated at temperature +6 ± 2 °C for 3 days, using Se (VI) containing solution (Se concentration 8.5 mg $L^{-1}$) or selenium with copper(II) containing solutions (Se concentration 8.5 mg $L^{-1}$, Cu concentrations 3 mg $L^{-1}$, 5 mg $L^{-1}$, 10 mg $L^{-1}$). After that sample were dried in the oven for 24 hours at temperature of + 73–108 °C. Control sample-germinating rye grain without microelements additives. Activity of amylase was determined in all experimental samples, because it characterizes the malt quality. Amylases are starch hydrolysing enzymes; more over there are known several amylases: α-amylases, β-amylases, isoamylases, etc. with different mechanisms of reaction. There different analytical methods were used for determination of α-amylase activity. The first was Ceralpha method (Megazyme test kits). The second method use complete reagents for quantitative determination of α-amylases (Phadebas Amylase Test). The third was iodometrical method. Different amounts of malt fortified with Se and Cu were added for investigation of rye flour technological properties. The falling number and the maximum viscosity were determined. The obtained results show that analysed additives of microelement copper decreases the enzyme activity. Analysed rye flour technological properties were better using malt only with selenium supplement.

**Key words:** selenium, copper, rye malt.

## INTRODUCTION

Rye (*Secale cereale* L.) is a major crop in Russia, Poland, Germany and the Scandinavian countries, where it is the major bread grain. Rye is also used to produce crisp bread and alcohol, and it is used as animal feed (McKevith, 2004). In Latvia a popular tradition is to use this grain for baking rye bread (Gailîte et al., 2013). Substantial quantities are also used as raw material in the distilling industry. There are some specialty beers in Germany and Austria made from rye malt (Hübner et al., 2010). Rye malt is the dried product of rye germinated under controlled conditions and is widely used in the production of bread, food flavoring, as ingredient for bakery products and as

color additives in the preparation of caramel. During germination (i.e. hydrothermal treatment in ambient conditions) the biosynthetic potential of grain is exploited and a number of hydrolytic enzymes are synthesised (Kaukovirta-Norja et al., 2004). Amylases play a significant role in grain germination and it is a key quality parameter in the commercial utilization of most cereals (Muralikrishna & Nirmala 2005). In germinating grain, starch degradation is initiated by α-amylase. α-amylase produces soluble oligosaccharides from starch, which are then hydrolysed by β-amylase to liberate maltose. Finally, α-glucosidase breaks down maltose into glucose (Yamasaki, 2003).

Many methods for the assay of α-amylase are described in the literature. The simplest and most direct is Ceralpha method (Megazyme test kits), using Complete reagents for quantitative determination of α-amylases (Mccleary et al., 2002). Other determination method of α-amylase activity is Phadebas Amylase Test, but for total amylase activity iodometrical method can be used. The falling number and the maximum viscosity characterize rye flour technological properties (Buchanon & Nicholas 1980; Cauvain & Young 2009; Antoņenko et al., 2014).

Selenium (Se) and copper (Cu) is essential microelements for animals and humans, they have also been found to be beneficial to plants (Vinit-Dunand et al., 2002; Weinstein et al., 2011; Fenga et al., 2013). Trace amounts of Se can promote growth in a variety of plants and protect plants from biotic and abiotic stress. Se may activate antioxidant system by elevating glutathione peroxidase (GPX) expression under abiotic stress conditions such as ultraviolet radiation, and low temperature (Wang et al., 2012), drought, water, salinity and heavy metals (metalloids) (HMs) (Fenga et al., 2013). Besides, supply of Se protects plants from a variety of herbivores and pathogens (Wang et al., 2012). Numerous researches show the effects of Se on wheat, barley and oat sprouting activity and positive influence on biologically active substances and high vitamins concentration in germinated grain (Dūma, 2010; Antoņenko et al., 2013b) as well as the rye malt quality: content of malt extract, diastase activity, total phenols and selenium accumulation degree in rye malt (Antoņenko et al., 2014). Copper is an essential micronutrient for plants that is a component of several electron transport enzymes (Lombardi & Sebastiani 2005), it is a component of various proteins, and particularly those involved in both the photosynthetic (plastocyanin) and the respiratory (cytochrome oxidase) electron transport chains (Baro´n et al., 1995). In plants copper interacts with a wide range of physiological and biochemical processes in cells (Caspi et al., 1999). But it also induces toxicity at tissue concentrations slightly above its optimal levels (Lombardi & Sebastiani, 2005). For higher plants the tolerance limit of $Cu^{2+}$ is $10^{-6}$ mol $L^{-1}$ (Krylova & Vassiljeva, 2011).

There are many investigations about microelements metabolism and accumulation in plant, health benefits, phytoremediation and bio fortification, but information about Se and Cu influence on the rye malt amylase activity is scarce. The aim of this research was to investigate the influence of microelements selenium and copper on the rye malt amylase activity and flour technological properties.

## MATERIALS AND METHODS

### Plant material

The research object was rye grain (variety ´Kaupo´) from Ltd. ´Naukšēni´, harvested in 2013. Rye grain of 96% viability were soaked and germinated at

temperature 6 ± 2 °C for 3 days. Five samples of rye malt were prepared. Control sample (I) without microelement additives. The II sample contains sodium selenate (Se concentration 8.5 mg $L^{-1}$). Samples III, IV and V besides selenium (Se concentration 8.5 mg $L^{-1}$) contain copper sulphate (copper concentrations 3 mg $L^{-1}$, 5 mg $L^{-1}$ and 10 mg $L^{-1}$). After germination grains were dried in the oven for 24 hours at temperature of 73–108 °C and ground with laboratory mill fitted with a 0.4 mm sieve. Moisture of malt samples ranged from 7.2% till 8.9%.

**Determination of total amylases activity iodometrically**

Amylase activity was assayed on the basis of the starch-iodometric method according to Zurcher & Hadorn (1972) with some modifications. By definition one unit of amylase activity (AA) equals mass of degradation (g) during one hour and express per 100g sample.

For determination a representative portion of sample (0.2 ± 0.001 g) was accurately weighted in a 100 mL glass test tube, dissolved in distilled water (electrical conductivity 10 μS $cm^{-1}$), held for 10 min with occasional shaking at room temperature and finally centrifuged. 20 mL from supernatant was placed in 50 mL test tube, in another test tube - 20 mL 0.5% starch solution. After holding in thermostat for 15 min at 40 °C both samples were mixed. After 0, 2, 6, 12, 20 and 30 minutes one mL of solution was put in 50 mL test tube with 30 mL of phosphate buffer solution with pH = 7.0 (58.9 g $Na_2HPO_4$ and 3.7 g citric acid dissolved in distilled water and filled till 1L) and 5 mL 0.05M iodine solution, filled with distilled water till 50 mL.

The total amylases activity (AA) was determined spectrophotometrically by absorption measurements at 565 nm and calculating according to the equation (1.):

$$AA = \frac{500 \cdot (A_0 - A_{30})}{A_0} \quad (1)$$

where: $A_0$ – absorption at t = 0 min; $A_{30}$ – absorption at t = 30 min.

**Determination of diastase activity with Phadebas**

The unit of diastase activity, the Gothe unit, is defined as that amount of enzyme which will convert 0.01 gram of starch to the prescribed end-point in one hour at 40 °C under the conditions of test. Results are expressed in Gothe units per gram of malt.

The diastase activity of samples was measured with the Phadebas method. A tablet of an insoluble blue-dyed, cross-linked starch was used as the substrate for the degradation reaction. After dissolving 1.00 g of malt in the 0.1M acetate buffer (pH = 5.2) in a volumetric flask, 5.0 mL of the malt solution was transferred to the test tube and incubated in a water bath at 40 °C for a few minutes. A blank was prepared by adding 5.0 mL of the 0.1M acetate buffer (pH = 5.2) solution and was treated in the same manner as a sample solution. After placing the Phadebas tablets into both test tubes, a timer was started. The tubes were quickly removed from the water bath, stirred and then returned to the water bath. After 30 min, the reaction was terminated by adding 1.0 mL of 1 M sodium hydroxide solution. The mixture was stirred again and filtered. The absorbance of the sample was measured at 620 nm with deionised water as a reference. The absorbance of the blank was subtracted from that of the sample solution ($DA_{620}$).

The diastase activity, expressed as DN or diastase number, was calculated according to the equation (2):

$$DN = 28.2 \times \Delta A_{620} - 2.64^{\vee} \quad (2)$$

where: $\Delta A_{620}$ – sample absorbance – blank absorbance

Diastase activity was referred to as DN in the Schade scale, which corresponds to the Gothe scale number, or g, of starch hydrolysed per hour at 40 °C per 100 g of malt (Sak-Bosnar & Sakac, 2012).

**Determination of α-amylase activity**

α-amylase activity (AA) was measured using 'non-reducing-end blocked p-nitrophenyl maltoheptaoside' as substrate and following instructions from the Megazyme. One unit of amylase activity is defined as the amount of enzyme required to release one micromole of p-nitrophenol from p-nitrophenyl maltoheptaoside in one minute. This unit is termed a Ceralpha Unit (units $g^{-1}$). α-amylase activity was calculated according to the equation (3):

$$AA = \Delta A_{410} \cdot 382 \quad (3)$$

where: $\Delta A_{410}$ – sample absorbance – blank absorbance.

**Viscosity measurements**

For analyzing falling number and viscosity the rye malt was added to rye flour (malt concentration in sample 1.25%). To determine the Falling Number, 7 g of flour are heated with 25 ml of distilled water in a water batch for one minute to approximately 95 °C. The viscosity of the starch gel thus obtained is then determined by measuring the time the stirring rod takes to sink through the gel to the bottom of the measuring cylinder. The Falling Number is the sum of the stirring and sinking time. It is stated in seconds. The falling number and amylogram curve (80 g flour sample) were determined by methods 56–81B and 22–10, respectively. Peak viscosity was obtained from the amylogram curve in Brabender units (BU). The amylograph value or peak viscosity is inversely correlated with amylase activity.

**Statistical analysis**

The results (mean values, standard deviation, *P*-value) were processed by mathematical and statistical methods. Significance was defined at $P < 0.05$. Statistical analyses were done using standard Microsoft Excel software.

## RESULTS AND DISCUSSION

**Determination of total amylases activity iodometrically**

The total activity of amylases was determined iodometrically. Obtained results showed that microelement selenium has positive influence on the amylases activity (Fig. 1). The relative increase of enzyme activity was for 13.4% compared with the control sample. Analysing the role of another microelement – copper, we can conclude that presence of copper decreases enzyme activity (Fig. 1, samples III–V). The highest

decrease of amylases activity was observed analysing samples with copper concentration 5 and 10 mg $L^{-1}$ (sample IV and V) – for 16% and 23% respectively. It can be explain with different antagonistic or synergistic relationships between microelements (Siedlecka, 1995; Ouzounidou et al., 1997).

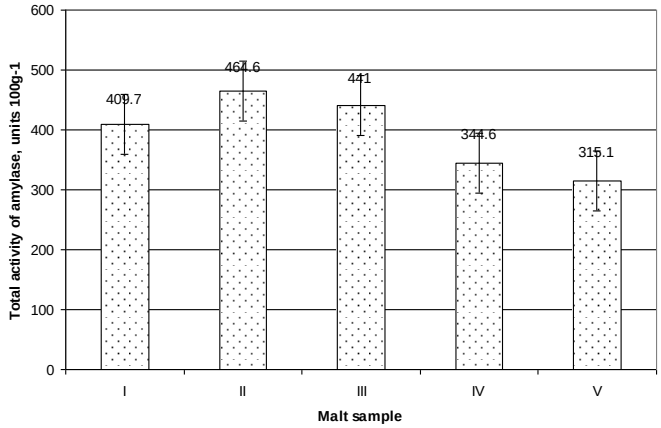


**Figure 1.** Total activity of amylases in rye malt samples. I – control; II – Se (8.5 mg $L^{-1}$); III – Se (8.5 mg $L^{-1}$) and Cu (3 mg $L^{-1}$); IV – Se (8.5 mg $L^{-1}$) and Cu (5 mg $L^{-1}$); V – Se (8.5 mg $L^{-1}$) and Cu (10 mg $L^{-1}$).

It was interesting to compare the activity of barley and rye malt. The obtained results (Fig. 2) showed that amylases in barley malt 'start to work' after 6 minutes from the beginning of reaction but enzymes in rye malt the maximum of activity reached only after 30 minutes.

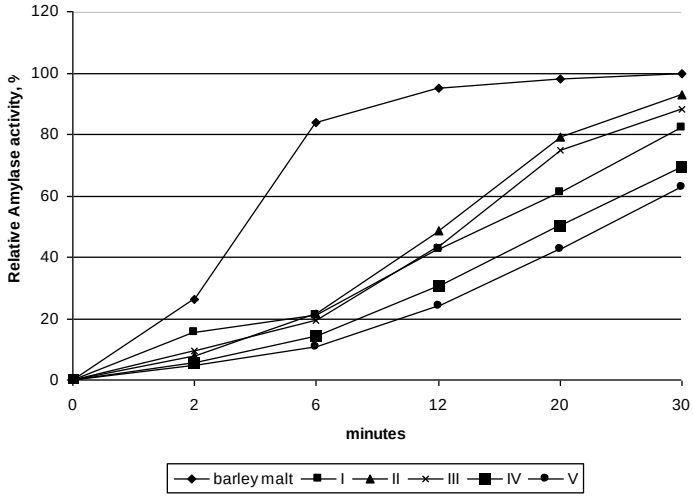


**Figure 2.** Relative amylase activity (%) in barley and rye malt. I – control; II – Se (8.5 mg $L^{-1}$); III – Se (8.5 mg $L^{-1}$) and Cu (3 mg $L^{-1}$); IV – Se (8.5 mg $L^{-1}$) and Cu (5 mg $L^{-1}$); V – Se (8.5 mg $L^{-1}$) and Cu (10 mg $L^{-1}$).

The results of research (Fig. 2) show evidence that the presence of selenium (sample II) promotes the activity of enzyme and this effect start to appear after 6 minutes from the beginning of reaction.

**Determination of α-amylase activity with Megazyme test kid**

There are several enzymes reacting as starch hydrolases, but the most important is the α-amylase. Determination of α-amylase activity with Megazyme test kid was used to be sure that the microelement copper has influence on the α-amylase activity. For determination of α-amylase, the well-known Ceralpha method was used. The results of research (Fig. 3) show that the highest activity of α-amylase was determined for second sample – rye malt with selenium additives. In this case the enzyme activity was two times higher comparing with the control sample. Therefore we can conclude that microelement selenium promoted enzyme activity and the changes were significant ($P < 0.05$). Numerous studies have demonstrated the Se beneficial effect including growth promoting activities (Terry et al., 2000), have shown enhanced resistance to certain abiotic stresses, drought (Kuznetsov et al., 2003), salinity, chilling and UV-radiation (Valkama et al., 2003).

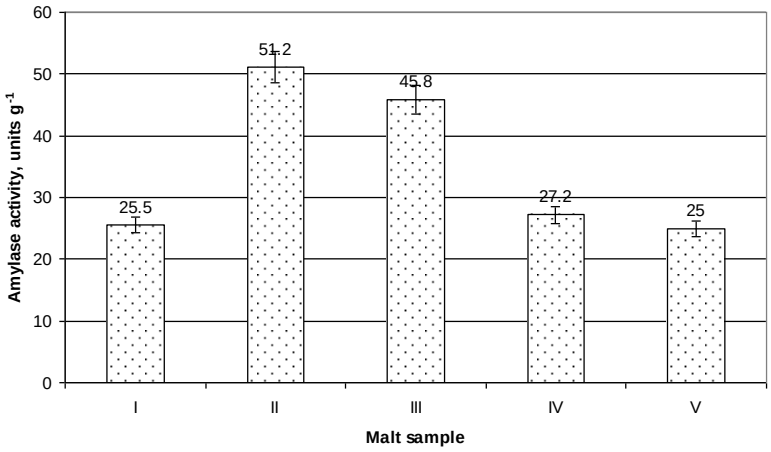


**Figure 3.** Determination of α-amylase activity with Megazyme test kid. I – control; II – Se (8.5 mg $L^{-1}$); III – Se (8.5 mg $L^{-1}$) and Cu (3 mg $L^{-1}$); IV – Se (8.5 mg $L^{-1}$) and Cu (5 mg $L^{-1}$); V – Se (8.5 mg $L^{-1}$) and Cu (10 mg $L^{-1}$).

Analysing the activity of α-amylase in rye malt with two different microelement additives, a conclusion can be drawn that a negative influence of microelement copper was observed. All analysed copper concentrations, except Cu 3 mg $L^{-1}$, significantly reduce the effect of selenium. If the concentration of copper is higher than 3 mg $L^{-1}$, the promotion effect of selenium was not observed. It can be explained with the copper toxic influence on the enzyme biosynthesis. From previous studies (Antoņenko et al, 2013a; Mihoub et al., 2005) it is known, that copper may inhibit biological processes in grain during germination. Further Cu in excess is strongly phytotoxic and may alter membrane permeability, chromatin structure, protein synthesis, enzyme activities, photosynthetic

and respiratory processes, and may activate senescence (Vinit-Dunand et al., 2002). The obtained results show similar tendency as previous results (Fig. 1).

**Determination of enzyme activity with Phadebas test**

There are several determination methods for enzyme activity. One of them is Phadebas Amylase test. Despite the fact that this method generally can be applied to honey samples, it may also be used for determination of diastase activity in another sample.

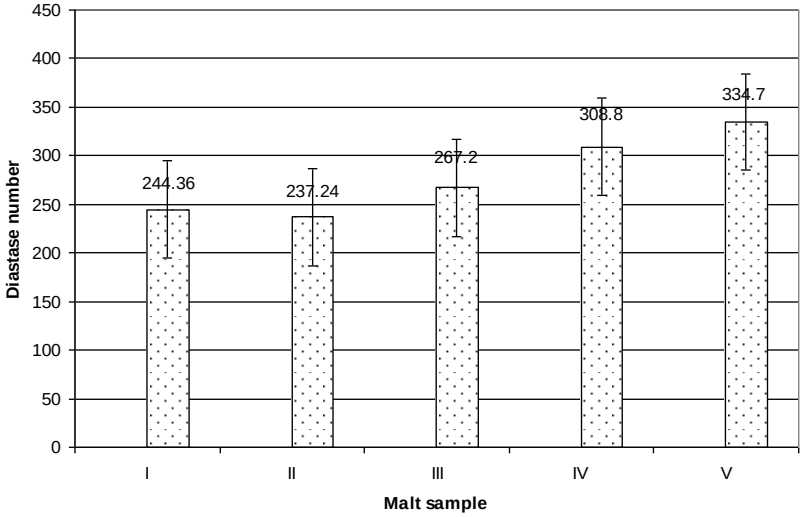


**Figure 4.** Determination of α-amylase activity with Phadebas test. I – control; II – Se (8.5 mg $L^{-1}$); III – Se (8.5 mg $L^{-1}$) and Cu (3 mg $L^{-1}$); IV – Se (8.5 mg $L^{-1}$) and Cu (5 mg $L^{-1}$); V – Se (8.5 mg $L^{-1}$) and Cu (10 mg $L^{-1}$).

The obtained results (Fig. 4) showed that presence of microelement selenium slightly decreases the enzyme activity (about 3%) comparing with the control sample, but selenium together with copper promoted the enzyme activity. The presence of Cu significantly ($P < 0.05$) increases the activity of α-amylase and the highest increase was observed at following microelements concentration: Se 8.5 mg $L^{-1}$ and Cu 10 mg $L^{-1}$. We can see that these experimental results are inversely proportional to results obtained with previously used methods. We think, that it can be explained by method's specificities. The final product of reaction is blue water soluble substance, determined photometrically at 620 nm. As it is known, copper compounds are also mainly in blue color and therefore it will influence the final results.

**The falling number and viscosity measurements**

Rye grain contains enzymes that attack all of its major constituents, and especially the starch-degrading amylases play a key role in relation to the baking quality of the flour (Seibel, Weipert 2001). To test the effect of different rye malt samples enriched with two microelements selenium and copper on the technological properties of rye flour, the flour samples with 1.25% additives of rye malt were prepared. The effects of

the rye malt, enriched with microelements were apparent in falling numbers and the results of amylogram (Table 1). The rye flour was used as control.

**Table 1.** The influence of microelements on the rye malt flour technological properties

| Sample | Falling number, s | Amylograph peak viscosity, BU |
|---|---|---|
| Rye flour | 200 | 616 |
| Rye flour + rye malt without additives | 171 | 553 |
| Rye flour + rye malt enriched with Se 8,5 mg $L^{-1}$ | 152 | 472 |
| Rye flour + rye malt enriched with Se 8,5 mg $L^{-1}$ and Cu 3 mg $L^{-1}$ | 160 | 570 |
| Rye flour + rye malt enriched with Se 8,5 mg $L^{-1}$ and Cu 5 mg $L^{-1}$ | 158 | 584 |
| Rye flour + rye malt enriched with Se 8,5 mg $L^{-1}$ and Cu 10 mg $L^{-1}$ | 176 | 599 |

Falling number (FN) and amylogram characterize the properties of starch and α-amylase activity. The greatest decrease in viscosity and falling number often corresponds to the high activities of hydrolytic enzymes in rye grain (Salmenkallio-Marttila & Hovinen, 2005). It is known that the rye flour normally has a FN in the range of 150 s, and in dry years the FN are 300 s and higher (Seibel & Weipert 2001; Hansen et al. 2004), the minimum FN requirement for intervention of rye in the EU was 100 s. Hansen, 2004 reported, if the FN is too low results in pasty and unacceptable bread.

The obtained experimental results confirm these conclusions. Different rye malt additives decrease falling number from 12% till 24%. The lowest falling number was determined for flour sample containing rye malt enriched with selenium. Taking into account previous experimental results it is evident that rye malt with selenium additives has the highest enzyme activity. These results also show that microelement copper has negative influence on the baking quality of rye flour - falling number and peak viscosity increase comparing with the control sample. The higher the concentration of copper, the greater increase of viscosity was observed.

## CONCLUSIONS

The additives of microelement selenium significantly increase the total activity of amylases (iodometrically determination) and activity of α-amylase (determined with Megazyme test) in rye malt. Using two microelements Cu and Se for rye malt production, the activity of amylases decreases, moreover, the higher the concentration of copper, the less activity was observed. Phadebas Amylase test gave inverse results, because the presence of Cu significantly increases results. Analysing technological properties of rye flour with rye malt additives, the lowest falling number and viscosity were determined for samples enriched with selenium. The malt samples enriched with copper increase falling number and viscosity that means lower amylase activity.

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# Extraction of biologically active compounds from fruit, berry and grain Grist using ViscoStar 150L enzyme complex

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**Abstract.** This work aimed to evaluate the efficiency of treating plant tissue with enzymatic agent ViscoStar 150L for the extraction of biologically active compounds. In the current study the screening of extraction methods from citrus fruit peels (grapefruit, lemon, orange) was performed. The samples treated with enzymatic agent ViscoStar 150L showed better extraction results than the traditional ethanol/water extraction method. Citrus peels' extracts assayed for antioxidant activity (determined as ferric reducing antioxidant power – FRAP) decreased in the following order: grapefruit > orange > lemon. The enzymatic agent ViscoStar 150L proved to have a positive synergic effect on juice yield from cowberry previously treated with a complex pectolytic enzymatic agent. The enzymatic agent ViscoStar 150L proved to have a synergic effect on grain grist mashes previously treated with an amylolytic enzymatic agent, the inhibitor activity of the compounds produced by actinomycetic microorganisms grown on substrates based on these mashes being higher than that of previously known inhibitors.

**Key words:** ViscoStar 150L, cowberry, citrus peel, grain grist, antioxidants.

## INTRODUCTION

Enzymes are *in vivo* process catalysts characterized by selectivity and specificity. An enzyme with absolute specificity would catalyze the reaction of one substrate only, while group-specific enzymes would catalyze similar process of a group of related compounds. For polyvalent substrates, or systems comprising multiple substrates, the optimal strategy would be using sets of enzymes targeted at different compound types.

Although the source of many enzymes is plant and animal tissue, controlled condition growth on various substrates makes use of the enzyme producing capacity of microorganisms. In bioengineering, enzymatic agents that contain multiple enzymes produced by sole controlled condition grown microorganisms are often used. ViscoStar 150L is one such enzymatic agent, produced by controlled condition grown *Trichoderma longibrachiatum* fungi, containing various cellulases and proteases.

Over the course of several decades the stable tendencies in food engineering have been reclaiming raw materials from by-products (Okwu & Emerike, 2007) and utilizing wildberry mass (Baraboï, 1984) due to their high biologically active compound content

(Ivanova, 1995; Rumiantseva et al., 2006; Zhilova, 2006; Bazarnova, 2007; Ovsiannikova et al., 2012; Liutikova, 2013). There is an increasing awareness of foodstuffs' antioxidant capacity, certain components of said foodstuffs being able to prevent biopolymer oxidation *in vivo* by scavenging active oxygen forms (Meliauskas et al., 2004; Temerdashev et al., 2006, Pantelidis et al., 2007). The addition of antioxidant components of plant origin to the food products is one of the ways to prolong the products' shelf-life due to the inhibition of the oxidation processes (Bazarnova, 2010). Citrus fruit and berry processing is known to yield significant amounts of antioxidants (Baraboi, 1984; Okwu & Emerike, 2007).

Citrus fruits are among the top consumer choices for fresh fruit. For processed citrus products, juices are in the lead. The peel, being the by-product of juice production, can still be recycled into dried dice, marshmallow, and citrus oils. Long shelf life products such as dried dice and jams retain significant P vitamin activity, the value usually associated with citrus juice (Grandall, 1977; Braddock, 1995). Citrus peel contains versatile biological agents such as flavonoids that improve metabolism, prevent atherosclerosis by improving blood vessel elasticity and exhibit antioxidant activity (ChongDe Sun et al., 2005; Zia-ur-Rehman, 2006; Jayaprakasha et al., 2008; Boshtam, 2011; Jabri karoui & Marzouk, 2013).

Cowberry is a valuable wildlife resource for the Northwest Russia and the Baltic states. Cowberry's nutritional and immunity modulating values are caused by its high content of carbohydrates, organic acids, vitamins, tannins, pectins and trace elements. The phenolic compounds contained in cowberry contribute to capillary wall strength, inflammation and atherosclerosis control and antioxidant capacity (Menshhikova et al., 2012; Liutikova, 2013).

Using starchy raw materials in the non-traditional role of nutrient suppliers for various biologically active compounds producing microorganisms (Akulova & Selezneva, 1995)

Claiming, or extracting, said biologically active compounds can be carried out in various ways (maceration, digestion, percolation, and others) usually involving: aggressive physical, such as ultrasonic, electro-thermic and electro-flotative, and chemical destructive techniques and reagents; prolonged exposure to same, resulting in time consumption; and unsatisfactory extraction specificity.

Enzyme treatment, however, does not carry any of these negative effects, the sole disadvantage being limited affordability (Schobinger, 2004).

Taking into account the diverse chemical nature of citrus peel, wildberry and grain grist components, extraction of said components should be aided by a complex enzyme solution or several solutions (Ovsyannikova et al., 2012; Pekhtereva et al., 2012). The extracts that would result, rich in various biologically active compounds, could be applied to multiple tasks related to food production.

By using biocatalysis it is possible to break down grain raw materials to the carbohydrates which are substrata for microorganisms that are biologically active substances' producers (Sharova, 2015).

## MATERIALS AND METHODS

The following enzymes and enzyme solutions were used in the current study.

ViscoStar 150 L (ENMEX, S. A. de C. V. 'Tarchomin Pharmaceutical Works 'POLFA' S.A.', Poland) enzyme complex contained several hemicellulases derived from controlled condition grown micromycetic *Trichoderma longibrachiatum* fungi.

Fructozym MA (ERBSLÖH Geisenheim AG, Germany) enzyme complex contained several pectinases, as well as other glycosidases, derived from controlled condition grown ascomycetic *Aspergillus niger* fungi.

*Aspergillus awamori* glucoamylase (EC 3.1.2.3, 1,4-α-D-glucan glycohydrolase) (Shandong Longda Bioproducts Co., Ltd., China).

Thermozyme 1000 L (ENMEX, S. A. de C. V.) enzyme solution contained α-amylase derived from controlled condition grown *Bacillus subtilis*.

Amylosubtilin (Sibbiofarm, Russia) contained α-amylase derived from controlled condition grown *Bacillus subtilis*.

Pancreatin (Biosintez, Russia) contained α-amylase derived from pig pancreas.

*Saccharomyces cerevisiæ* invertase (EC 3.2.1.26, β-D-fructofuranesidase) (Biolar, Russia).

The citrus fruits used in the experiments were commercially available in St.-Petersburg, Russia, and devoid of sort specificity. Ester oils were purchased from a retail drug vendor in St.-Petersburg, Russia.

Citrus extract preparation**.** Peels were crushed in a worm-type crusher. Extraction liquid was fed into weighed citrus peel samples and left for 30 ± 5 minutes; then the extract was separated by centrifugation**.** The following conditions were used for the experiment: t = **(**20 ± 2) °C; pH 7.0; dilution 1:10 (9 parts of liquid to 1 part of raw product mass, $^{v}/_{w}$).

The spectral characteristics of the extracts were examined through a Shimadzu UV-1800 spectrophotometer. All the spectroscopy sample solutions were prepared by diluting 1 ml extract by 24 ml distilled water in calibrated 25 ml flasks.

The concentration of ascorbic acid in the resulting liquid was assessed using the Murry titration method based on the ability of ascorbic acid to reduce 2,6-dichlorophenyl indophenol (DCIP) in an acidic medium (Chupakhina, 2000). Crushed peels were ground in a mortar with an addition of quartz sand while 5% hydrochloric acid was being gradually added until the consistency became paste-like. The mortar and the pestle were rinsed with 5% HCl and the rinse-offs and the ground peels diluted with 5% HCl in calibrated 100 ml flasks. Upon filtration, 10 ml of the solutions were then pipetted into Erlenmeyer flasks and titrated with a 0.001n DCIP solution until a lasting (no less than 30 seconds) rose coloration was obtained. The concentration of ascorbic acid was then calculated using the formula

$$C = a \cdot 0.088 \cdot V_1 \cdot 100 \cdot b^{-1} \cdot (V_2)^{-1} = a \cdot 8.8, \quad (1)$$

where: C – ascorbate concentration, mg $dm^{-3}$; a – amount of DCIP spent on titration, ml; 0.088 – coefficient of resolution for pure ascorbate; b – peel sample mass, g; $V_1$ – extract volume (100 ml); $V_2$ – clarified solution volume titrated (10 ml).

Content of phenolics in the extracts was assessed by the Folin-Ciocalteu colorimetric method using Folin reagent produced by Fluka (Dienisienko, 2015). Calibrated 25 ml flasks

were used for mixing 12.5 ml of sample liquid, 2.5 ml of Folin reagent, 7.5 ml of 20% (%$_w$) sodium carbonate and sufficient amount of distilled water to raise the resulting volume to 25 ml. The optical density values (D) were read on a Shimadzu UV-1800 (Shimadzu, Japan) spectrophotometer at 760 nm after a settling time of 20 minutes.

Determination of the total antioxidant activity (AOA) of extracted compounds. Total AOA of the extracts was determined using a modified ferric ion reducing antioxidant power method – FRAP with an indicator system of Fe(III)/Fe(II) – *o*-phenantroline (Temerdashev et al., 2006). Phenanthroline (NPF Ural Invest) and chloric iron (Rexant) were used. Optical density was assessed with a PEC N-57 photoelectric colorimeter with a 507 nm transmission peak light filter. After a cuvette (10 mm thick liquid slate) was used for mixing 0.3 ± 0.01 ml extract, 0.3 ± 0.01 ml 0.045M o-phenantroline solution, 0.3 ± 0.01 ml 0.025M $FeCl_3$ and 1.5 ± 0.01 ml 96% (%$_v$) ethanol, the optical density values were read after a settling time of 20 minutes. The readings were then resolved for pure ascorbate using a standard calibration curve.

Preparation of cowberry juice. Cowberry was puréed in a LMT-1 lab mill and 0.05% (%$_w$ wet mass) Fructozym MA enzyme solution was added to the purée with a 1 hour reaction time at 30 °C, then 0.01% (%$_w$ wet mass) ViscoStar 150L enzyme solution was added with a 1.5 hours reaction time at 50 °C. The juice was then drained by a lab press.

Cowberry and cowberry juice analysis was carried out according to respective Russian national standards unified with the AIJN Code of Practice.

The anthocyanin content was assessed by colorimetry using a PEC N-57 photoelectric colorimeter at $\lambda = 510$ nm. The colorimeter readings were then resolved for cyanidin-3 glycoside content 100 $g^{-1}$ raw material wet mass using a calibration curve.

Liquids' gravity was assessed with a PTR46 refractometer (Index Instruments) using methodology described in (Yermakov, 1972) and in a manner according to the user manual.

Rice flour and rye grist mashing technique was derived from (Sharova et al., 2012).

Examination of rice flour and rye grist mashes involved growing such strains as *Streptomyces lucensis* VKPM AS-1,743 (Sharova & Hodkievich, 2009) and *Streptomyces violaceus* VKPM AS-1,734 (Sharova et al., 2009) on substrates based on the mashes. Spectroscopy was used to compare corresponding inhibitory activity towards pancreatic α-amylase (EC 3.2.1.1; 1,4-α-D-glucan glycohydrolase) as described in (Sharova, 2015), Activity towards glucoamylase (EC 3.2.1.3) of *Aspergillus* was measured by the glucose oxidase method (Xiao et al., 2006). The inhibitors were prepared with a method previously described in (Sharova & Hodkievich, 2009).

The pH stability of the inhibitors was tested by comparing inhibitory activity before and after exposing 0.1% solutions of the inhibitors to a 0.1M universal buffer solution (pH 2–12) at (25 ± 1) °C for 180 minutes.

The temperature stability of the inhibitors was assayed based on the difference in inhibitory activity before and after exposing 0.1% solution of the inhibitors to 25–200 °C in distilled water for 180 minutes.

Inhibitor IR spectrums were examined with a Specord 75R spectrograph (Specord, Germany) in transmission mode: resolution 4,000, amplification 8.0×, mirror speed 0.6329, diaphragm 100.00, DTGSKBr detector, KBr beam splitter.

All experiments were performed with at least three replications. Statistical analysis was performed using Microsoft Office Excel tools with an assumed 95% confidence level.

## RESULTS AND DISCUSSION

### Citrus peel extraction and analysis

The principal value of citrus peel lies in high flavonoid content, resulting in an equally significant antioxidant value of the peel extracts (Devis, 1947; Bacco, 1998). Plant tissue extraction is a complex physical and chemical process. Plant cell wall is a lipid and carbohydrate complex layered on a rigid cellulose fibre underlay, resulting in structural strength as well as selective permeability. For the better effectiveness of biologically active compounds' extraction from vegetable raw material, enzyme treatment is commonly used to facilitate cell wall destruction. Qualitative assay of the biocatalysis efficiency in screening experiments was based on spectroscopic analysis. The flavonoid content assessment technique makes use of a short wave maximum in the UV absorption spectrums of flavonoid-rich solutions at 286 nm (ethanol/dimethyl sulphoxide 10:2). The prominence of the maximum in the spectrums of extracts derived from all the peel types allowed skipping $AlCl_3$ chelation before direct spectroscopy (Yevseieva, 2013). The absorption spectrums observed all had a maximum at $\lambda = 280$ nm (Fig. 1). The hypsochromic shift of the absorption maximum can be attributed to polarity alteration and solubilizing capacity of the solvent.

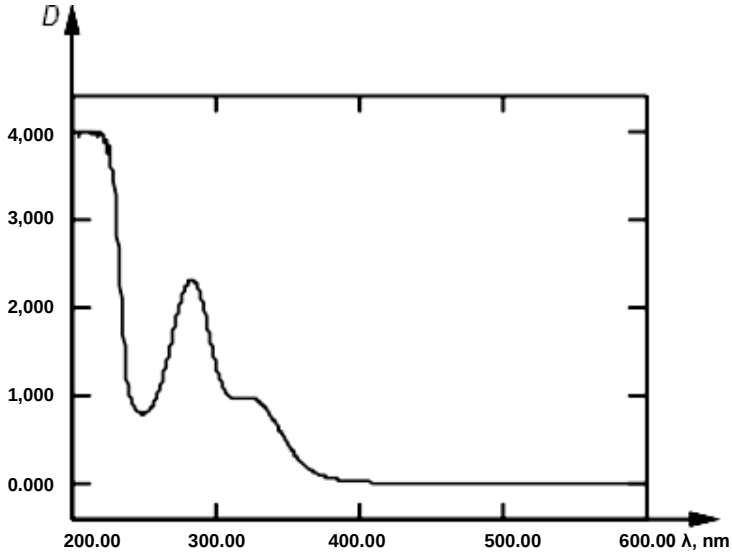


**Figure 1.** Grapefruit peel extract absorption spectrum.

Optical density (D) values in the respective UV absorption spectra were effectively used to compare extraction efficiencies of 60% ethanol/water (%v) solution to solutions with enzyme at different concentrations (Fig. 2).

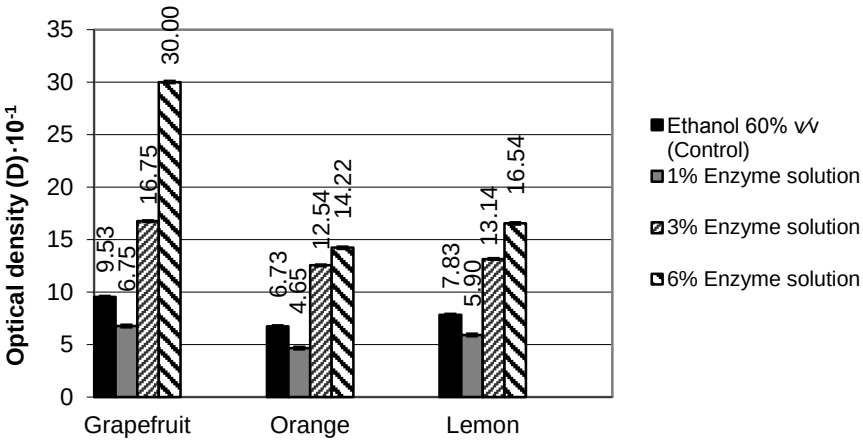


**Figure 2.** The effect of enzyme solutions on citrus peel extraction efficiency.

As shown in Fig. 1, the overall efficiency of optically active ingredients' extraction by means of enzyme treatment is better than that of the common ethanol extraction method. Additionally, as the concentration of the enzyme solutions gets higher, the extraction effectiveness gets better. One can see that the effect of enzymes is particularly noticeable in grapefruit peel extracts.

Further analysis of various peel extracts with 6% ViscoStar 150L concentration was carried out to determine the biologically active compounds' content and total antioxidant activity (AOA). AOA assay methods often involve significant reagent and time expenditure (Hasanov et al., 2004). The modified FRAP-based ferric ion reducing antioxidant power method allows direct assessment of low molecular antioxidant content (Benzie & Strain, 1996). The Fe(III)/Fe(II)−*o*-phenantroline indicator system performs express examination of liquids' reduction capacity which is an effective antioxidant potential indicator (Temerdashev et al., 2006).

The modified FRAP method was used to examine total AOA of fresh citrus fruit juices, the corresponding peel extracts and ester oils (Table 1). As one can see, the least AOA is demonstrated by the fruit juices, followed by the ester oils, and the most AOA is demonstrated by the peels, especially the grapefruit peel.

**Table 1.** Assessment of total AOA of extracts (E), citrus oils (EO) and juices

| | Grapefruit | | | Orange | | | Lemon | | |
|---|---|---|---|---|---|---|---|---|---|
| | E | EO | Juice | E | EO | Juice | E | EO | Juice |
| Ascorbate equivalent, mg ml$^{-1}$ | 0.40 ± 0.01 | 0.19 ± 0.02 | 0.09 ± 0.01 | 0.20 ± 0.01 | 0.12 ± 0.01 | 0.02 ± 0.00 | 0.18 ± 0.01 | 0.16 ± 0.01 | 0.04 ± 0.00 |

The data obtained contradict the results of a previously published study (Gorinstein et al., 2005) where orange peel was found to have more total AOA as per the modified FRAP method, but the contradiction may be attributed to sort-to-sort differences (Izosimova, 2004). A correlation has been found between TAA and the content of native oxidation protectors of the plant, such as ascorbic acid and phenols (Makarova et al., 2010).

Citrus fruits are characterized by high ascorbic acid retention in storage due to the absence of the ascorbate lyasing enzyme, ascorbate oxidase (EC 1.10.3.3.). According to ascorbic acid content assessment, the richest in ascorbic acid is the grapefruit peel extract, followed by the lemon peel (Fig. 3).

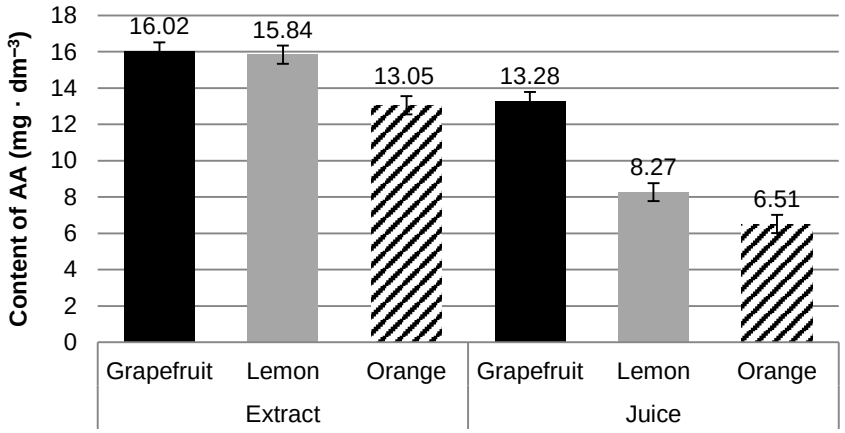


**Figure 3.** Ascorbic acid content of citrus peel extracts and citrus juices, $mg^{-1}$.

Total phenolic compound content was assessed via optical density (*D*) measurement after colouration by Folin reagent. The method is based on the ability of phenolics to reduce phosphotungstenate and phosphomolybdate contained in Folin reagent to indigo coloured oxides and reading the optical density values with a colorimeter.

Grapefruit and lemon peel extracts proved to retain more light at the given wavelength, which means they contain the most phenolic compounds (Fig. 4).

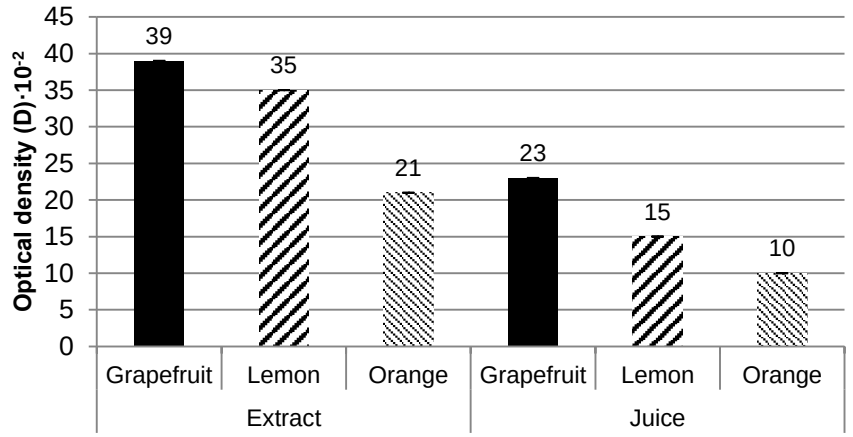


**Figure 4.** Optical density (*D*) of peel extracts and citrus juices with Folin reagent at 760 nm.

The results are theoretically predictable because an increase in the content of both ascorbic acid and phenolics must cause an AOA increase, but a clear correlation is not always found (Fedoseeva et al., 2008). This is believed to be the result of not only the

polyvalence of antioxidants in vegetative raw materials, but also of the difference in AOA assessment techniques. For instance, in one study (Gorinstein et al, 2005) ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) method tested TAC varied little between red and white grapefruit, while in another study (Anagnostopoulou, 2006) DPPH (2,2-diphenyl 1-picrylhydrazyl) method tested AOA proved to be much higher in red grapefruit. Additionally, some researchers are inclined to attribute TAC more to the ascorbate content and less to the phenolic content (Del Caro et al., 2004).

**Biocatalysis in cowberry juice preparation**

Analysis of lingonberries grown in Karelia (Table 2) and comparison of their indicators with the data presented in the literature (Izosimova, 2004) leads to the conclusion about the high nutritional and medicinal properties of cranberries growing in Karelia.

**Table 2.** Characteristics of the cowberry used in the experiment

| Material (place of growth) | Characteristics (wet mass all) | | | | |
|---|---|---|---|---|---|
| | Solids, Plato | Sugars, % $^{w}/_{w}$ | Organic acids, % $^{w}/_{w}$ | Ascorbate, mg $dm^{-3}$ | Anthocyans, mg $dm^{-3}$ |
| Cowberry (Karelia) | 9.9 ± 0.1 | 7.7 ± 0.3 | 1.8 ± 0.1 | 14.8 ± 1.2 | 264.0 ± 0.8 |

Various doses and types of enzyme solutions were tried out on cranberry purée to study the effect on juice yield (Table 3). As shown in Table 3, the use of Fructozym pectolytic enzyme complex allowed for 10% ($^{w}/_{w}$) more juice to be collected, while the addition of ViscoStar 150L solution as well as Fructozym increased the yield by further 10%.

**Table 3.** The effect of enzyme solutions on cranberry juice yield

| Sample number | Enzyme solution | Enzyme solution dosage, % $^{v}/_{w}$ wet mass | Treatment conditions Temperature, °C | Time, h | Juice yield, % $^{v}/_{w}$ wet mass |
|---|---|---|---|---|---|
| 1 | None (Control) | – | | | 65 |
| 2 | Fructozym MA | 0.05 | 30 | 0.5 | 72 |
| 3 | Fructozym MA<br>ViscoStar 150 L | 0.05<br>0.01 | 50 | 1.5 | 78 |

The various effects of enzyme solutions on the basic characteristics of cranberry juice are shown in Table 4.

**Table 4.** The effects of enzyme solutions on cranberry juice basic characteristics

| Sample number | Enzyme solution | Juice yield, % $^{v}/_{w}$ | Gravity, Plato | Anthocyans, % $^{w}/_{v}$ | Ascorbic acid, % $^{w}/_{v}$ |
|---|---|---|---|---|---|
| 1 | None (Control) | 65 | 10.2 ± 0.3 | 23.8 ± 0.6 | 1.0 ± 0.1 |
| 2 | Fructozym MA | 72 | 13.6 ± 0.2 | 24.6 ± 0.3 | 1.2 ± 0.1 |
| 3 | Fructozym MA<br>ViscoStar 150 L | 78 | 14.8 ± 0.2 | 25.4 ± 0.3 | 1.4 ± 0.2 |

Extraction of biologically active compounds from cranberries with the use of enzyme solutions allows for increase of juice yield, anthocyans and vitamin C content and can, therefore, be recommended for industrial-scale cranberry juice manufacturers.

**Biocatalysis in grain grist mashing**

According to various examinations (Akulova & Selezniova, 1995; Sharova & Hodkievich, 2009; Sharova et al., 2012; Sharova, 2015), actinomycetic bacteria grown on polysaccharide-based mediums produce inhibitors that show more activity towards amylases (α-amylase and glucoamylase) specific to macromolecular carbohydrates that are glycosidases, meaning that they hydrolyse glycoside bonds in starch molecules. α-amylase cleaves alpha bonds that are deep inside the molecule, effectively destructing the starch before glucoamylase hydrolysis may start that breaks individual glucose links off the terminus. α-amylase and glucoamylase thus both regulate the supply of glucose to the bloodstream that, if left uncontrolled, may lead to diabetes, obesity and other carbohydrate metabolism issues. At least one of the sources of these enzymes in animal (including human) bodies is believed to be the pancreas. Other known amylase sources are microorganisms. Therefore, it is important to test the inhibitors produced on common source amylases, not least on α-amylase derived from the body of an animal.

Glycosidase inhibitors are commonly produced by strains of Streptomyces, such as *Streptomyces violaceus* VKPM AS-1,734 and Streptomyces lucensis VKPM AS-1,743.

To study further the prospective applications of ViscoStar 150L for the extraction of biologically active compounds from vegetative raw material, the following experiment was conducted.

In the experiment rice flour and milled rye (with various pulverization ratios) were treated with ViscoStar 150L and Thermozyme 1,000L enzyme solutions during mashing to produce suitable substrates for microbial amylase inhibitor production. The effect of ViscoStar 150L on the composition of these substrates and subsequent changes in inhibitor yield from bacteria grown on these substrates was then examined. Pancreatic α-amylase derived from the pancreas of a pig as well as various microbial amylases were used as test samples for inhibitory activity assessment. The choice of pig pancreas amylase is based on the similarity between the biochemical processes in the body of a pig with said processes in the human body.

It has been shown that in the case of rice flour mash peak accumulation of inhibitors showing maximum activity towards pancreatic α-amylase requires the medium to contain 75–80% $^{w}/_{w}$ dextrins and 20–25% $^{w}/_{w}$ oligosaccharides (glucose, maltose).

In the mash of rye grist pancreatic α-amylase inhibitory activity proved to be 1.5–2 times lower (Table 5).

It has been shown that amylase inhibitors produced on rice flour mash substrate possess inhibitory activity towards other amylases, such as *B. subtilis* α-amylase, *Aspergillus awamori* glucoamylase and *S. cerevisiæ* invertase, even if 3–4 times lower than the 'target' activity.

Amylase inhibitors produced on rye mash substrate do not show inhibitory activity towards microbial glucoamylases. It may be attributed to a significantly different inhibitor producing microorganisms' substrate composition.

**Table 5.** Starchy raw materials hydrolyzing inhibitory activity

| Strain name | Grist mashed | Inhibitory activity in mash, $cm^{-3}$, towards | | | |
|---|---|---|---|---|---|
| | | pig pancreas α-amylase | *B. subtilis* α-amylase | *Aspergillus awamori* glucoamylase | *S. cerevisiæ* invertase |
| *Streptomyces lucensis* VKPM AS-1,743 | Rice flour | 1,200 ± 50 | 400 ± 15 | 250 ± 80 | 450 ± 25 |
| | Rye grist | 600 ± 50 | 240 ± 20 | – | 380 ± 10 |
| *Streptomyces violaceus* VKPM AS-1,734 | Rice flour | 1,100 ± 50 | 350 ± 15 | 250 ± 80 | 410 ± 12 |
| | Rye grist | 600 ± 50 | 130 ± 20 | – | 180 ± 10 |

The inhibitory activity towards pancreatic α-amylase assessed after rice flour and rye grist centrifugate fermentation was 1.5 times higher in comparison to the results of non-centrifuged hydrolysates (1,600–1,800 units of inhibitory activity per $cm^{-3}$). The centrifugation removes high molecular mass compounds of protein and carbohydrate-protein nature that slow down the growth, development and biosynthetic activity of *Streptomyces*. Membrane filtration was used to clarify the cultural liquids of *Streptomyces* and to produce isolated solutions of pancreatic α-amylase inhibitors.

According to IR-spectroscopy, the inhibitors produced are carbohydrates in nature and contain α-1,2- and α-1,4-glycoside bonds, double bonds, carbonyl, hydroxyl, =NH and $–NH_2$ prosthetic groups (Fig. 5).

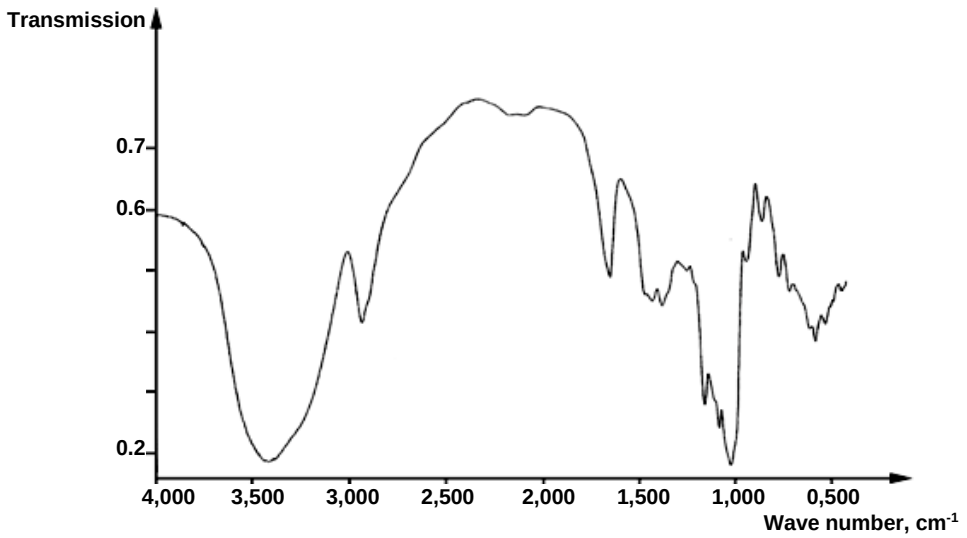


**Figure 5.** Pancreatic α-amylase inhibitor spectrogram.

The IR spectra of the inhibitor from Streptomyces lucensis VKPM AS-1,743 and inhibitor from *Streptomyces violaceus* VKPM AS-1,734 contained intensive bands characteristic of stretching and deformation vibrations and vibrations of the double bonds. Oscillations in intervals of change of the wave number (ν) were observed: 3,450–3,400 $cm^{-1}$ (hydroxyl group, imines group and amino group associated); 3,100–2,900 $cm^{-1}$ (methyl group); 1,750–1,600 $cm^{-1}$ (aldehyde group, a double bond); 1,400–1,200 $cm^{-1}$ (hydroxyl group, deformation); 1,200–1,000 $cm^{-1}$ (acetylene group),

850–700 $cm^{-1}$ (amino group, deformation). Deformation and skeletal vibrations of polyatomic systems are in the region of spectrum below 1,500 $cm^{-1}$. Oscillations of α-1,4 and α-1,2 glycoside bonds (ν = 756, 934 and 938 $cm^{-1}$) were observed. The data of IR spectra testify to the similarity of the structures of the inhibitor from *Streptomyces lucensis* VKPM AS-1,743 and inhibitor from *Streptomyces violaceus* VKPM AS-1,734.

The inhibitors produced supposedly retain their activity throughout a wide temperature (20–200 °C) and pH (2–12) spectrum. The inhibitors may serve as the substance for medicinal forms and food additives for prevention and treatment of diabetes, obesity and other carbohydrate metabolism issues.

## CONCLUSIONS

Biocatalysis by ViscoStar 150L enzyme solution allows for a 2–3-fold increase in the effectiveness of biologically active compounds' extraction from citrus fruit peels. Peel extracts demonstrate higher total antioxidant capacity compared to fresh juices and ester oils. Extracted ascorbic acid and flavonoid content is also higher than that of juices, both the highest in grapefruit.

The use of Fructozym and ViscoStar 150L solutions in cranberry juice preparation allowed for a 20% ($^{w}/_{w}$) increase in juice yield, a 14.5% increase in gravity, a 10% increase in anthocyan and a 13.6% increase in vitamin C content.

The activity of inhibitors produced by microorganisms cultivated on mediums derived from grain grists retain almost 100% of their activity throughout a wide temperature and pH spectrum. The data obtained do not contradict previously known microbial pseudo-saccharide enzyme inhibitor properties and show possibilities for use of biologically active compounds derived from grain mashes as substance for food additives.

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# The application of green tea Extract as a source of antioxidants in the processing of dairy products

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**Abstract.** Regular consumption of foods containing antioxidants reduces the bodily content of free oxygen radicals, which can cause pathological changes and premature organism aging. The aim of this work was the development of the formulations and determination of the parameters for the production of cottage cheese products with polyphenol fraction of green tea extract as a source of plant antioxidants. Parameters to obtain extracts with the high content of extracted substances and high antioxidant activity were determined. Optimal performance was achieved by brewing dry green tea leaves with (70 ± 2) °C water, followed by steeping at the same temperature for 10 minutes with continuous mechanical stirring. Optimal dry tea leaves to water ratio used for tea extracts' preparation was identified. The level of tea extract in cottage cheese products' recipes was determined. The flavour fillers which combine the best with green tea extract and taste were identified. The positive effect of tea extract component on shelf life of cottage cheese product was shown.

**Key words: A**ntioxidant activity, polyphenols, the parameters to obtain extracts, cottage cheese products.

## INTRODUCTION

Multiple studies conducted in different countries indicate that one of the reasons for early aging and many diseases is the oxidation stress, excessive concentration of free oxygen radicals in the physiological fluids of the body (Menshikova et al., 2006; Carlsen et al., 2010). Some adverse external factors can cause the level of active oxygen forms to rise. These forms can provoke damage of the macromolecules, which would cause the disruption of metabolic processes (Vladimirov & Archakov, 1971; Meerson, 1981).

To prevent the effects of the oxidation stress, it is necessary to consume products containing antioxidants, which can reduce the effects of free radicals. Bioflavonoids are one of the leading groups of antioxidants, which have anti-cancerogenic, anti-sclerotic, anti-inflammatory and hypoallergenic properties (Grek, 1999; Menshikova et al., 2006) and bactericidal activity (Gordon & Wareham, 2010, Stalnaja et al., 2014). Preventive application of antioxidants, especially during stress, increases general body resistance (Sorokina et al., 1997).

Main sources of bioflavonoids (polyphenols) for humans are drinks (tea, juices, wine), fruit and vegetables. Significant amount of phenol compounds are found in various tea breeds. The level of free glycolized and polymerized flavonoids reaches 30−40 mg $l^{-1}$ in drinks brewed according to the standard procedure (Manach et al., 2005). Polyphenols not only affect biological activity but also organoleptic properties, in particular colour and taste of plant raw materials.

One of the leading products with the highest content of water-soluble antioxidants is green tea (Fedoseeva et al., 2008). In non-fermented green tea 30−40% of dry weight is represented by phenol compounds which are mainly kachetins. Phenol compounds in green tea prevent consequences of oxidative stress (Nakagawa et al., 2002; Fedoseeva et al., 2008) have bactericidal (Gordon & Wareham, 2010) and anticarcinogenic activity (Lukin, 2015).

High antioxidant activity of polyphenols in body is manifested at low concentration of weaker synergists. In human body synergists are represented by reduced energy substrates (NADH and NADPH) formed during main catabolic processes and essential nutrients entering body with food (vitamins C, E, K, carotinoids, microelements) (Sorokina et al., 1997; Sharapova, 2008). In food products fortified with native plants, the components of food may be synergists.

Application of plant extracts assists in the extension of functional products' variety. This group includes such products as balsamic syrups (Pekhtereva, 2004), dairy drinks (Zabodalova et al., 2014), fermented whey-based drinks (Lazareva & Vysokogorsky, 2007; Palagina & Prikhodko, 2010), etc.

At the same time, the addition of antioxidant components of plant origin to the food products, including dairy, is one of the ways to prolong the products' shelf-life due to the inhibition of the oxidation processes (Bazarnova & Veretnov, 2004; Lazareva & Vysokogorsky, 2007; Bazarnova, 2010; Brosalin et al., 2013).

In dairy industry such antioxidants as tocopherols, salts of gallic and ascorbic acids, synergic antioxidants, lecithins and dihyrdoquercetin (DHQ) are used. For instance, DHQ is used in sour cream, yogurt, processed cheese, condensed milk, etc. for shelf life extension. Addition of DHQ at 0.02% of total fat enables extending dairy products' shelf life two to three times (Lazareva & Vysokogorsky, 2007; Palagina, 2010; Veretinskaya, 2011). Technology for the manufacture of fortified cottage cheese using antioxidant OriganoxWS (Frutarom) and dietary fibre complex Steyd Milk B-01 has been developed, optimal dose of the antioxidant being 0.03% (Ponomarev et al., 2011a; Ponomarev et al., 2011b).

Cottage cheese is one of the most popular protein dairy products. In spite of numerous examples of plant-derived components' application for the manufacture of dairy product, research in cottage cheese is limited to the addition of fruit fillers (Ostroumov et al., 2003; Capajeva & Suchkova, 2014) and commercial food additives based on them (Reshetnik et al., 2011).

The objective of this work was to develop the composition and identify technological parameters for the manufacturing of cottage cheese product using polyphenol fraction of green tea extract as the source of plant antioxidants.

## MATERIALS AND METHODS

The objects of the study were green tea extracts and the samples of cheese products developed with them. Tea used in the study was Ahmad Tea (Nanchang Ltd).

**Evaluation of mass fraction of dry substances**

Mass fraction of dry substances in aqueous extracts of green tea and in whey was evaluated by accelerated drying. Two layers of gauze were placed to the bottom of weighing bottle, dried with no lid in exsiccator at 105 °C for 20–30 min, followed by closing the lid and cooling in exsiccator for 20–30 min and weighing. 3 $cm^2$ of product studied was placed to the prepared weighing bottle, spread evenly on the gauze surface, followed by weighing with the lid closed. Next, open weighing bottle and the lid were placed to the exsiccator at 105 °C for 60 min, followed by closing the bottle, cooling and weighing. Drying and weighing was conducted after 20–30 min until the difference between two consequent weights was below 0.001 g. Mass concentration of dry matter DS was calculated as follows (1):

$$DS = (M_1 - M_0)\ 100 / V, \quad (1)$$

where: $M_1$ – weighing bottle with gauze and product before drying, g; $M_0$ – weighing bottle with gauze and product after drying, g; $V$ – product volume, ml.

**Determination of density**

The density of the aqueous tea extracts was determined at 20 °C using aerometer type AOH-1 with the limit of absolute error 1.0 kg $m^{-3}$.

**Evaluation of active acidity**

The active acidity of extracts was evaluated by potentiometric method using pH-meter pH-410. Sample extract amount of (40 ± 5) $cm^3$ was placed to the 50 $cm^3$ glass at (20 ± 2) °C, followed by immersing electrode pair into the glass.

**Determination of water-soluble antioxidants' activity**

Total antioxidant activity (TAA) in tea extracts was determined using FRAP method (Ferric Reducing Antioxidant Power) with indicator system Fe (III)/Fe (II) − o-phenanthroline (Temerdashev et al., 2006). Phenanthroline (NPF Ural Invest) and chloric iron (Rexant) were used. Optical density was measured using photoelectric colorimeter 'FEC H-57' (Medical Eng., light filter with transmission peak λ = 507 nm). The extract amount of 0.3 ml was placed to cuvette with light path of 1 cm followed by addition of 0.3 ml of 0.045 M o-phenanthroline solution and 1.5 ml of 96% ethanol. 30 min after the addition of 0.3 ml of 0.025 M $FeCl_3$, indication of the instrument was read. TAA was calculated as an ascorbic acid equivalent using standard calibration curve.

**Manufacturing of cheese products**

The test and control samples of cheese products were manufactured from fresh cow milk. Milk was heated to 40−45 °C followed by separation using electric separator Motor Sich CMS-80 to cream and skim milk with fat level below 0.05%. Skimmed milk was pasteurized at 76−78 °C with loading for 20 s, followed by cooling to 28−30 °C. For coagulation the following components were added into the milk: dairy starter cultures of

*Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris, Lactococcus lactis subsp. lactis biovar diacetilactis*; calcium chloride; rennet powder.

The reagents were added to the mixture in the following quantities: starter – 2% $^{v}/_{v}$; solution of calcium chloride on the basis of addition of 400 g of anhydrous salt per 1,000 kg of mixture (as 40% aqueous solution); rennet powder – 1 g with activity of 100,000 units per 1,000 kg of mixture (as 1% aqueous solution).

The mix was agitated and left for fermentation in thermostat at 28−30 °C. The curd obtained was pressed to remove whey to moisture level of 80%. The defatted cottage cheese was a control sample and was used as a base for cottage cheese products with green tea extract.

**Preparation of green tea extract for the fortification of cottage cheese product**

Tea sample of 28 g was placed to a glass container, followed by addition of 100 g of water at (70 ± 2) °C and exposure for 10 min in water bath at (70 ± 2) °C with continuous agitation. Next, the extract was filtered and cooled down to (20 ± 2) °C.

**Preparation of cottage cheese product fortified with green tea extract**

In order to obtain final product with moisture level of 80%, defatted cottage cheese was preliminarily pressed. The moisture content in the cottage cheese after the compression was calculated considering the amount of tea extract that will be added as well as the mass fraction of dry substances in the extract and original cottage cheese product according to the formula (2):

$$W_1 = 100 - (100 \cdot DS_1 - D\,DS_2) / (100 - D), \quad (2)$$

where: $W_1$ – moisture content in the cottage cheese product after the compression, %; $DS_1$ – mass fraction of dry substances in the cottage cheese product, %; $DS_2$ – mass fraction of dry substances in the tea extract, %; $D$ – a dose of the tea extract, %.

The amount of whey which must be separated during compression was calculated by the formula (3):

$$M_2 = M_3 (DS_4 - DS_3) / (DS_4 - DS_5), \quad (3)$$

where: $M_2$ – the mass of separated whey during the compression, kg; $M_3$ – mass of the original cottage cheese product, kg; $DS_3$ – mass fraction of dry substances in the original cottage cheese product, %; $DS_4$ – mass fraction of dry substances in the cottage cheese product after the compression %; $DS_5$ – mass fraction of dry substances in the whey, %.

Green tea extract was added to preliminarily pressed defatted cottage cheese at a certain level and mixed with tea extract using blender.

**Preparation of cottage cheese product fortified with green tea extract and fruit fillers**

Fruit fillers were added to the pressed defatted cottage cheese fortified with green tea extract at the level of 15%. Experimental and control samples packed to glass jars with screwed metal lids were stored in refrigerator at (4 ± 2) °C.

**Evaluating of titratable acidity**

The cheese products were evaluated for titratable acidity. The method is based on neutralization of acids in the product with 0.1 mol·dm$^{-3}$ sodium hydroxide solution with

phenolphthalein indicator. 5 g of product was placed to porcelain mortar, agitated thoroughly and ground with pestle. Next, 50 $cm^3$ water at 35−40 °C was added with small portions followed by 3 drops of phenolphthalein addition. The mixture was agitated and titrated with alkaline solution till pink colour appearance was stable for 1 min. Acidity in Turner degrees (°T) was calculated by multiplication of volume ($cm^3$) of sodium hydroxide solution used for titration by 20.

**Determination of moisture level in cottage cheese**

Moisture level in cottage cheese was determined using moisture analyzer Elex-7. Preliminarily single-layer filter paper bags 150 × 150 mm were prepared as follows: a bag was diagonally folded, a corner was turned up by 15 mm and enclosed to parchment of a slightly bigger size with no edges folding. Ready bags were dried in the analyzer between metallic plates heated to 102–105 °C for 3 min followed by cooling and storing in exsiccator. Ready bags were weighed with an error below 0.01 g, followed by placing 5 g of product, weighing it with an error below 0.01 g. Bags with product were closed, placed to the analyser between metallic plates heated to 102–105 °C and exposed for 5 min. Bags with dried samples were cooled in the exsiccator for 3−5 min and weighed.

Moisture level in the product $W_2$, % was determined using equation (4):

$$W_2 = (M_4 - M_5)\ 100/5, \quad (4)$$

where: $M_4$ – bag weight before drying, g; $M_5$ – bag weight after drying, g; 5 – sample weight, g.

**Examination of organoleptic characteristics**

Organoleptic evaluation was conducted by 5-point scale using sensory analysis method (Kantere et al., 2001). The samples were evaluated by a trained panel of 12 members. Twelve panelists (age 22–38 years) qualified for sensory evaluation techniques and regular consumers of products estimated the sensory properties of the samples.

**Microbiological analysis**

Lactic acid bacteria (LAB) number, yeasts and moulds and colibacillus number were determined in the cottage cheese product during refrigerated storage.

The number of LAB in cottage cheese products was determined using plate method. Nutrient broth used was agar with hydrolyzed milk. For inoculation dilutions $10^{-6}$–$10^{-9}$ were used. Inoculates were incubated at (30 ± 1) °C for 3 days followed by counting of LAB colonies' number and re-calculation per 1 g of product.

The number of yeasts and moulds was determined by inoculating of cottage cheese products diluted to $10^{-1}$ on Petri dishes with wort agar. Plates were incubated at 20−23 °C for 5 days. Microorganisms were classified as yeasts and moulds based on characteristic growth on the nutrient broth and cell morphology. Colony numbers of yeasts and moulds were counted separately.

Method of coliform count determination was based on the ability of coliform bacteria to ferment lactose and acid formation at (37 ± 1) °C for 24 h on the nutrient broth. An indicator of coliform growth on Kessler medium is the formation of gas in the float. Inoculation of product on Kessler medium was conducted for dilutions $10^{-1}$, $10^{-2}$ and $10^{-3}$. 1 $cm^3$ of each dilution was inoculated to the tube with 5 $cm^3$ liquid Kessler

medium with floats. Tubes with inoculates were placed in thermostat at (37 ± 1) °C for 24 h, followed by examination and visual determination of presence or absence of gas in the floats.

All experiments were performed with at least three replications; data was processed by methods of mathematical statistics with 95% confidence level. Confidence interval was calculated according to standard procedure using Student coefficient for confidence level of 0.95.

## RESULTS AND DISCUSSION

### Preparation of green tea extracts

Preparation of green tea extract is crucial for its application as an ingredient of cottage cheese product. Extract components can fulfil various functions and perform as quality identifiers, deliver consumer quality attributes, including organoleptic properties. For instance, sugars, acids, salts and other tastings affect taste, colourings affect colour, flavourings affect flavour. In order to select the optimal way of preparing green tea extract, a comparative study of the effects of brewing conditions and exposure was conducted for physico-chemical and organoleptic properties of tea extracts. Selection of brewing temperature of (70 ± 2) °C was based on the data on the loss of useful properties of green tea at higher temperatures (U Vey Sin, 2005). This is related to bioflavonoids' instability. Parameters varied are given in Table 1.

**Table 1.** Technological parameters of tea extracts' preparation, brewing temperature (70 ± 2) °C

| Method No | Temperature of exposure, °C | Exposure duration, min | Agitation |
|---|---|---|---|
| 1 | 70 ± 2 | 5, 10, 15 | – |
| 2 | 20 ± 2 | 5, 10, 15 | – |
| 3 | 20 ± 2 | 5, 10, 15 | + |
| 4 | 70 ± 2 | 5, 10, 15 | + |

### Properties of extracts

Comparative evaluation of extracts' properties was done for the following parameters: dry matter level, density, pH and antioxidant activity. Change of these characteristics reflects the efficiency of the process of transferring of substances from tea extracted under different brewing conditions. As Fig. 1 shows, when increasing the exposure duration in all tea brewing methods, the acidity values of tea extracts (pH) tended to decline.

The tendency is positive since it indicates more active transfer of organic acids – soluble compounds of tea leaves – to the extract. The extracts of tea brewed by methods 1 and 4 had the lowest pH values.

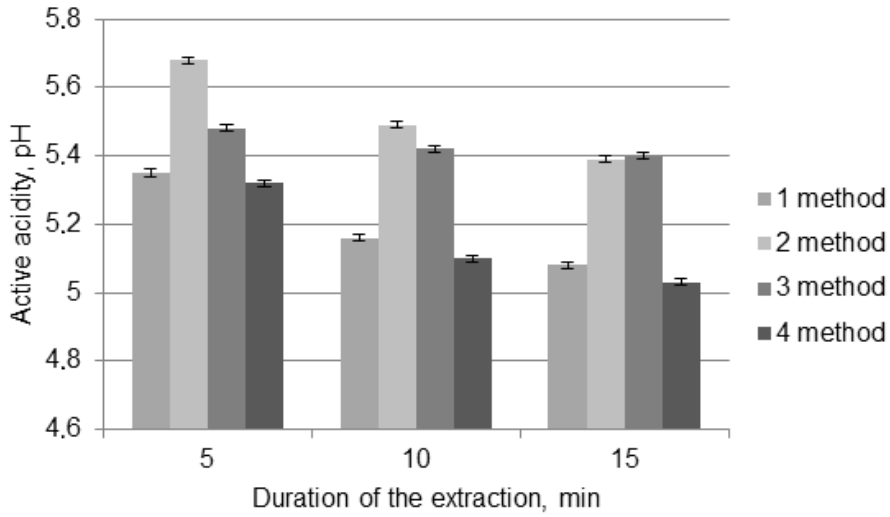


**Figure 1.** Active acidity of tea extracts.

Comparative evaluation of dry matter levels in extracts showed that methods 1 and 4 are more preferable (Fig. 2). Increasing the exposure duration in all brewing methods resulted in increased dry matter levels.

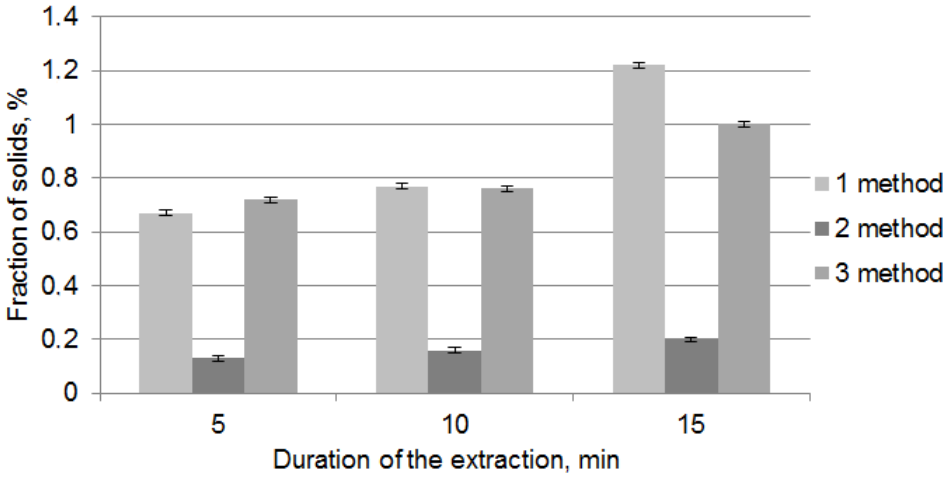


**Figure 2.** Dry matter level in tea extracts.

Determination of antioxidant activity of tea extracts revealed the advantage of method 4 (Fig. 3). However, it was noted that increasing exposure over 10 min is unreasonable since for the extract with maximal antioxidant activity obtained in methods 1 and 4, it leads to decline of this parameter. Apparently it is linked with the instability of antioxidants at elevated temperatures.

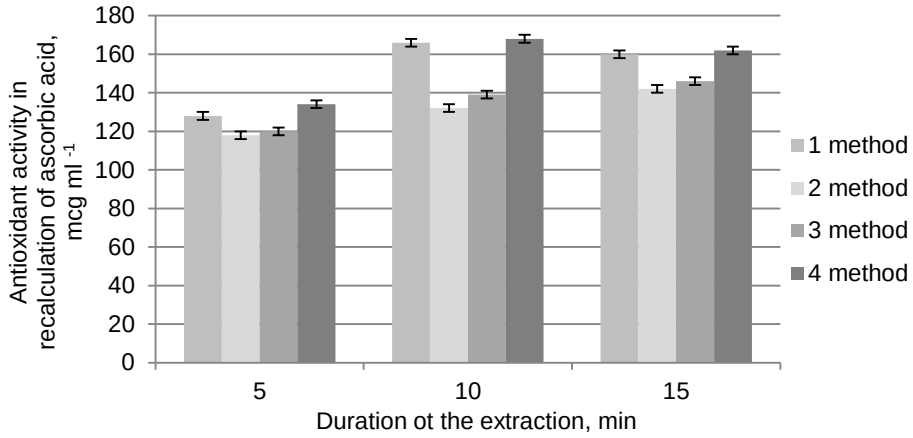


**Figure 3.** Antioxidant activity of tea extracts.

Organoleptic properties of the tea extracts agree with their physico-chemical properties. Exposure in water bath at brewing temperatures was accompanied with increased taste and flavour in the tea extract. Agitation during exposure intensified the process insignificantly.

Thus, brewing dry tea leaves followed by exposure in water bath at (70 ± 2) °C for 10 min with continuous agitation appears to be the most reasonable method of tea extract's preparation. This method enables to obtain extracts with the highest level of extractive compounds and antioxidant activity.

Addition of tea extracts to cottage cheese product was conducted after preparation of cottage cheese base with lowered moisture level. Addition of the green tea extract to normalized mix leads to a loss of tea components during syneresis together with whey.

It is reasonable to use the tea extract with maximal level of dry matter. Dry matter level in tea extract can be increased by elevating tea amount during brewing. The effect of extraction hydromodule (dry tea to water ratio) in the range 1:25 to 1:2.5 (tea amount from 4 g to 40 g per 100 g water) on extract's properties was studied. Brewing was conducted using method 4. Physico-chemical properties of extracts are given in Table 2.

**Table 2.** Study of extract propertied at different dry tea levels

| Dry tea levels (g per 100 g of water) | pH | Density, kg $m^{-3}$ | Dry matter, % | Antioxidant activity in ascorbic acid equivalents, $\mu g \cdot ml^{-1}$ |
|---|---|---|---|---|
| 4.0 | 5.29 ± 0.02 | 1001 ± 2 | 1.2 ± 0.2 | 162 ± 3 |
| 8.0 | 5.24 ± 0.02 | 1003 ± 1 | 2.1 ± 0.1 | 169 ± 2 |
| 12.0 | 5.19 ± 0.01 | 1008 ± 3 | 3.3 ± 0.2 | 176 ± 3 |
| 16.0 | 5.16 ± 0.01 | 1013 ± 2 | 4.5 ± 0.3 | 187 ± 3 |
| 20.0 | 5.14 ± 0.01 | 1018 ± 2 | 5.8 ± 0.2 | 197 ± 1 |
| 24.0 | 5.12 ± 0.01 | 1021 ± 1 | 6.5 ± 0.3 | 206 ± 2 |
| 28.0 | 5.11 ± 0.01 | 1024 ± 1 | 7.3 ± 0.2 | 209 ± 3 |
| 40.0 | 5.02 ± 0.02 | 1033 ± 2 | 9.6 ± 0.1 | 210 ± 3 |

Ten times increase in dry tea level resulted in 3% increase of dry matter in extracts and 5% decline in active acidity. Antioxidant activity in ascorbic acid equivalent was 29.6% higher. Most considerable changes in the majority of parameters analyzed (mainly dry matter and antioxidant activity) were noted when increasing dry tea level till 28 g per 100 g water. Further elevation of dry tea level was accompanied by decline in dynamics of change.

Observed trends reflect change in extract components' ratio. Since the purpose of green tea extract's addition was not only to obtain cottage cheese product with new sensory properties but also to increase shelf life, antioxidant activity was considered as the most important physico-chemical parameter. Thus 28 g of green tea per 100 g was selected as the most reasonable level.

**Cottage cheese enrichment with tea extracts and taste fillers**

Sensory analysis plays an important role in the evaluation of food quality. Sensory analysis conducted based on scientific ground excels some laboratory methods especially in terms of such parameters as taste, flavour and texture. For products such as wine and tea, organoleptic (sensory) analysis happens to be the only way to determine quality so far. Sensory characteristics enable to determine how the product is perceived by consumer. The disadvantages of organoleptic analysis include subjectivity of evaluation, relative expression of its results in dimensionless units, incomparability and insufficient reproducibility of results. In order to reduce the disadvantages mentioned, it is necessary to train panelists of basic organoleptic parameters for evaluation and to follow the conditions of organoleptic analysis. Taste perception plays the most important role in organoleptic analysis of foods (Nikolaeva 2006; Shepeleva, 2008).

The influence of green tea extract level on the organoleptic parameters of cottage cheese product was studied. Green tea extract has a specific taste and flavour. It was required to determine the maximal extract level which enables to obtain product with best organoleptic characteristics.

Cottage cheese samples with various green tea extract levels and taste fillers were evaluated. Green tea extract with dry matter level $(7.3 \pm 0.2)\%$ to defatted pressed cottage cheese at the level of 1 to 17% (1% interval) was added and mixed using a blender. Tea taste was not detected or was considered too weak at the levels up to 7% (inclusively) in the cottage cheese. At the levels of 8% and 9%, the cottage cheese product had a pleasant moderately expressed green tea taste and flavour. Such a product can be recommended for manufacture without extra taste fillers. In cottage cheese samples with extract levels from 10% to 16%, an increase of bitter taste was observed and correction with taste fillers was required. In the sample with 17% extract, tea taste was described as 'too bitter, unpleasant'.

At the next stage, taste fillers were selected. Right choice of taste fillers enables to preserve the pleasant tea taste in the cottage cheese product while neutralizing the bitter taste. Moreover, various taste fillers and their compositions enable to extend variety and obtain new products with original taste characteristics.

The following taste fillers were used: sugar, chocolate syrup, rosehip syrup, cranberry syrup, lemon juice, raspberry jam, honey, pine nuts, walnuts and raisins. The selection of filler was performed according to criteria such as compatibility with tea taste, neutralization of bitter tea taste and cost. Results of the evaluation are given in Fig. 4.

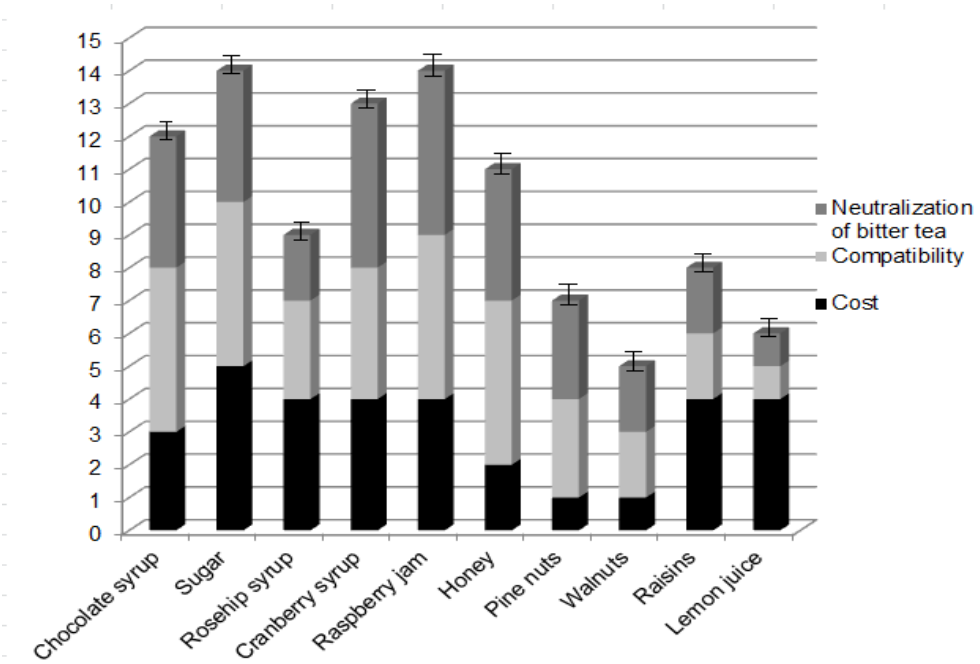


**Figure 4.** Organoleptic and cost analysis of cottage cheese products with taste fillers.

Evaluation of each criterion was done using a 5-point scale. Higher value for taste evaluation meant better organoleptic parameters: 0 – not perceived, 1 – somewhat perceived, 2 – weak intensity, 3 – moderate intensity, 4 – strong intensity, 5 – very strong intensity. In terms of cost, the higher was the taste filler's cost, the lower was the score.

Cottage cheese samples with cranberry syrup, raspberry jam, honey, sugar and chocolate syrup obtained the highest scores. These fillers can be used both individually and in combination. The selected fillers were varied by their dose of adding.

Organoleptic evaluation of test samples was conducted. Such analytical descriptors as tea taste, taste filler, bitter taste, colour and consistency were used. Each parameter was evaluated using a 5-point scale. Score increase for each parameter except for 'bitter taste' indicated product improvement. By contrast, the higher the 'bitter taste' score was, the lower was the perception of it.

As an example, the results of organoleptic evaluation of cottage cheese product with tea extracts (15%) and chocolate syrup are given in Fig. 5.

It was noted that the higher was the filler level in the cottage cheese product, the lower the bitter taste was and the stronger chocolate taste was perceived. When chocolate syrup was added above 19% level, tea taste became less strong which resulted in score decline for this descriptor. Filler quantity level did not have noticeable influence on the colour of cottage cheese product, whereas texture remained different at chocolate syrup levels up to 19% inclusively.

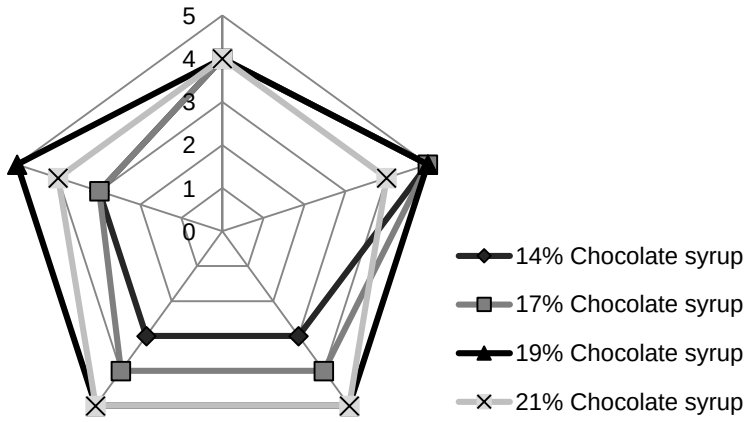


**Figure 5.** Organoleptic parameters of cottage cheese products with chocolate syrup.

The following taste fillers for cottage cheese product with green tea extracts are recommended: 19% chocolate syrup; 11% raspberry jam and 4% sugar; 12% honey; 14% cranberry syrup and 5% sugar.

The study of the cottage cheese with green tea extract was conducted. Defatted cottage cheese was prepared. First half of it was used as a control sample; the second half was used to prepare the product with 9% green tea extract.

Control and test samples were placed for storage at 0–4 °C. During storage acidity, organoleptic and microbiological parameters of cottage cheese were determined.

Acidity of green tea extract was lower than acidity of cottage cheese, thus acidity of the test sample was lower than in the control sample (Table 3). It was found that acidity of the sample with green tea extract increased by 6 °T during 10 days, whereas in control sample it increased by 22 °T. Elevation of acidity resulted from the formation of acid by microorganisms, thus the results indicate the bactericidal activity of green tea and the shelf life improvement of fortified cottage cheese product.

**Table 3.** Acidity of cottage cheese products during storage

| Shelf life, days | 0 | 1 | 3 | 5 | 7 | 10 |
|---|---|---|---|---|---|---|
| Control sample, °T | 220 ± 2 | 230 ± 1 | 234 ± 1 | 236 ± 1 | 238 ± 1 | 242 ± 2 |
| Test sample, °T | 204 ± 1 | 206 ± 1 | 206 ± 1 | 208 ± 1 | 208 ± 1 | 210 ± 1 |

The results of organoleptic evaluation of cottage cheese products indicate that change of taste properties in control sample during storage happened faster and more profoundly when compared to test sample (Figs 6 and 7).

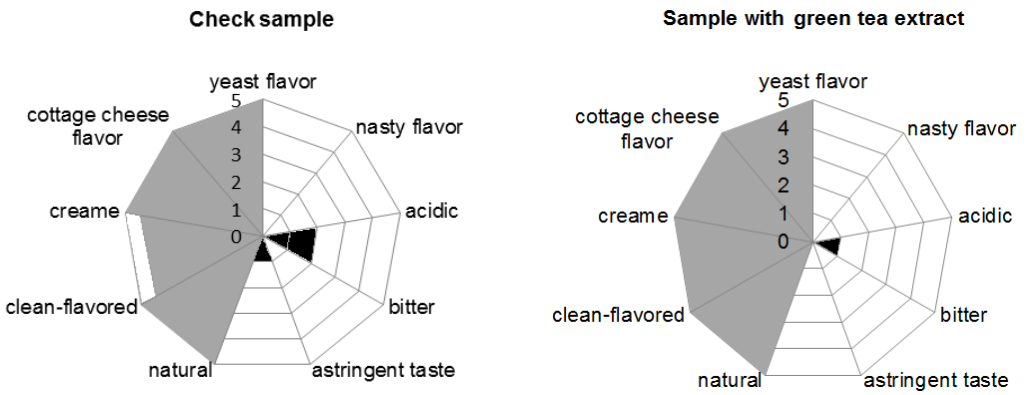


**Figure 6.** Taste scores in cottage cheese products after 7 days of storage.

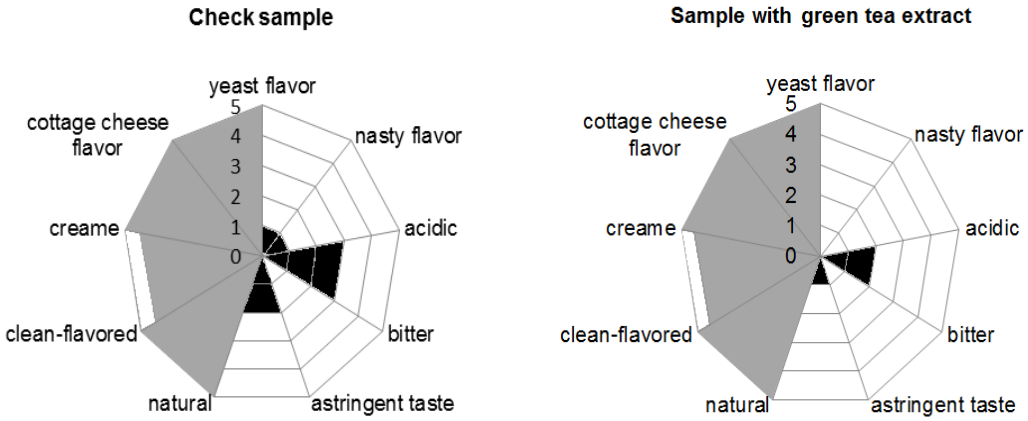


**Figure 7.** Taste score in cottage cheese products after 10 days of storage.

Microbiological parameters of cottage cheese products during storage are given in Table 4.

**Table 4.** Microbiological parameters of cottage cheese products during storage (0 – fresh sample; C – control sample, T – test sample with tea extract)

| Parameter | Limit | Storage duration, days | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | | 5 | | 7 | | 10 | |
| | | C | T | C | T | C | T | C | T |
| Lactic acid bacteria, CFU $g^{-1}$* | At least $10^6$ | $7\times10^7$ | $7\times10^7$ | – | – | – | – | $2\times10^6$ | $1\times10^6$ |
| Yeasts, CFU $g^{-1}$ | 100 | <10 | <10 | <10 | <10 | 40 | <10 | 110 | 40 |
| Mould, CFU $g^{-1}$ | 50 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 |
| Coliforms in 0.01 g | Not allowed | Not detected | | | | | | | |

* CFU $g^{-1}$ – colony-forming units in 1 g.

Lactic acid bacteria (LAB) number was determined in fresh samples (0) and at different points during storage (5, 7, 10 days). The slightly higher LAB number in control sample resulted in faster increase in acidity during storage.

During storage in both samples coliforms in 0.01 g were not detected, mould did not exceed 10 CFU $g^{-1}$. Yeasts' number in control sample till the end of shelf life did not exceed limit, whereas in test sample it was 40 CFU $g^{-1}$.

The study of control and test samples during storage showed that the addition of green tea extract to cottage cheese products increases their stability during storage. The results obtained agree well with the data of bactericidal properties of flavonoids (Stalnaja, 2014) and green tea (Lukin, 2015).

## CONCLUSIONS

Based on the conducted studies, the following conclusions have been made:

Technological parameters for the preparation of tea extracts with highest levels of extractive substances and antioxidant activities were established. Optimal parameters were achieved by brewing dry green tea leaves in water at (70 ± 2) °C, followed by exposure for 10 min with continuous agitation.

Optimal green tea leaves to water ratio was 28:100.

The tea extract level should be 9% for the preparation of cottage cheese product without taste fillers, whereas for products with taste fillers it should be 16%.

The following taste fillers for cottage cheese products with green tea extracts are recommended: 19% chocolate syrup; 11% raspberry jam and 4% sugar; 12% honey; 14% cranberry syrup and 5% sugar.

Positive effect of green tea extracts on shelf life of cottage cheese products was established.

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# Image analysis of the shapes and dimensions of Teff seeds (*Eragrostis tef*)

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**Abstract.** With aid of the image analysis using trio ocular microscope the dimensions, circumferences and areas in two perpendicular planes of Teff seeds were measured and based on this information the new ellipsoid model of the seed's shape was derived and compared with measured values. From statistical analysis implies that this model on probability 0.95 is significantly identical with measured values of the Teff seeds. Determined model can help more accurately set up and developed accurate mathematical model for describing mechanical behaviour of individual seeds as well as bulk seeds.

**Key words:** model, ellipsoid, ball, cereals, grain, dimension.

## INTRODUCTION

Teff (*Eragostis tef)* has been widely cultivated and used in Ethiopia and neighbourhood countries, it accounts for about a quarter of total cereal production in Ethiopia and it is main product used in local cuisine (Stallknecht, 1993; Bultosa & Taylor, 2004; Arendt & Zannini, 2013; Bultosa, 2016). However the physical and mechanical properties of Teff seeds are not very well described in professional literature yet (Zewdu & Solomon, 2007) which is given by very small dimensions of Teff seeds (less than 1 mm) (Gebremariam et al., 2014). Nowadays they are only few already published studies focused on the processing of Teff seeds (Stewart & Getachew, 1962; Stojceska et al., 2010; D'Silva et al., 2011). Understanding to the dimensions and shape of the seeds is important factor for creating mathematical models required for modelling of the seed's mechanical behaviour. For accurate mathematical model of the seed's shape that are used in modern simulation processes, for example finite element model (Petrů et al., 2012; Petrů et al., 2014), they are inappropriately ball models of the seeds based on the geometric mean diameter or arithmetic mean diameter (Lizhang et al., 2013; Zhan et al., 2013). From already published studies follows that mathematical model should be based on the ellipsoid model with respect to the true cross section areas and circumferences of the modelled seeds (Petrů et al., 2012; Petrů et al., 2014). The aim of this study is to determinate the dimensions of Teff seeds and with their utilization to derivate accurate ellipsoid mathematical model for the Teff seed shape.

## MATERIALS AND METHODS

### Sample

Teff (*Eragrostis tef*) seeds (Fig. 1) obtained from Hawassa region, Ethiopia, were used for the experiment. The moisture content $M_c = (11.4 \pm 0.8)\%$ d.b. of the samples was determined by the conventional method ASAE using a standard hot air oven with a temperature setting of 105 °C and a drying time of 17 h (ASAE S410, 1998). Samples having 100 g of weight were randomly selected from a batch of Teff seeds for determining moisture content. The mass of each sample was determined using an electronic balance (Kern 440–35, Kern & Sohn GmbH, Balingen, Germany). Three samples were tested for each experiment and the results averaged.

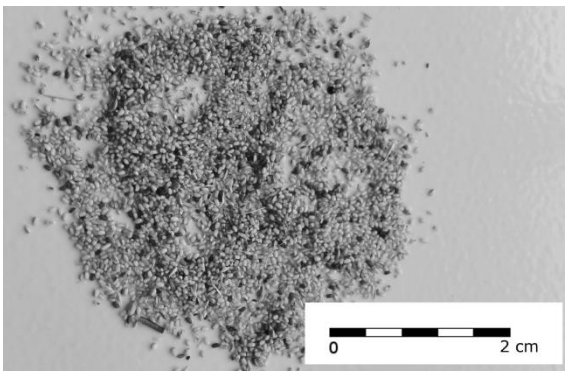


**Figure 1.** Teff seeds.

### Seeds dimensions

For determination of dimensions 20 pieces of Teff seeds were used. Dimensions of the each seeds, length $L^*$ (mm), width $W^*$ (mm), thickness $T^*$ (mm), areas $S_I^*$ ($mm^2$), $S_{II}^*$ ($mm^2$) and circumferences $O_I^*$ (mm), $O_{II}^*$ (mm) were determined by digital image analyses using ImageJ software from pictures which were taken with aid of trio ocular microscope (Bresser BioScience Trino, Besser GmbH, Rhede, Germany) in two perpendicular planes as shown in Fig. 2.

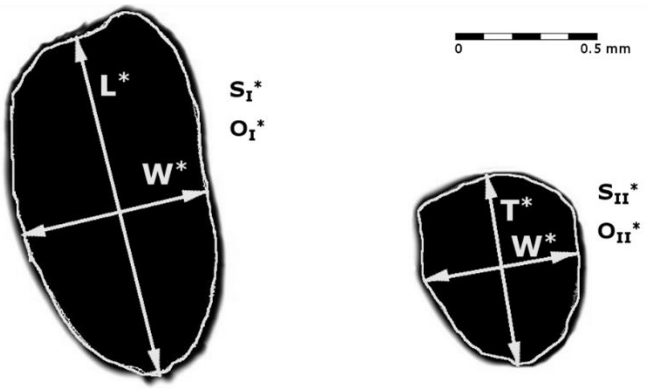


**Figure 2.** Image analysis of Teff seed.

Geometric mean diameter $D_g$ (mm) and arithmetic mean diameter $D_a$ (mm) were calculated using the following equations (Eq. 1 ; Eq. 2), (Mohsenin, 1970)

$$D_g = \sqrt[3]{W^* \cdot T^* \cdot L^*} \quad (1)$$

$$D_a = \frac{W^* + T^* + L^*}{3} \quad (2)$$

Sphericity was calculated by Eq. 3 (Mohsenin, 1970).

$$\phi = \frac{D_g}{L^*} \quad (3)$$

Area based on arithmetic diameter $S_a$ ($mm^2$) and area based on geometric diameter $S_g$ ($mm^2$) were calculated by Eq. 4 and Eq. 5.

$$S_a = \frac{1}{4} \cdot \pi \cdot D_a^2 \quad (4)$$

$$S_g = \frac{1}{4} \cdot \pi \cdot D_g^2 \quad (5)$$

**Theory and modelling**

Model of the Teff seeds shape was based on the similarities with ellipsoid (Fig. 3) and on that principle the basic assumptions were determined.

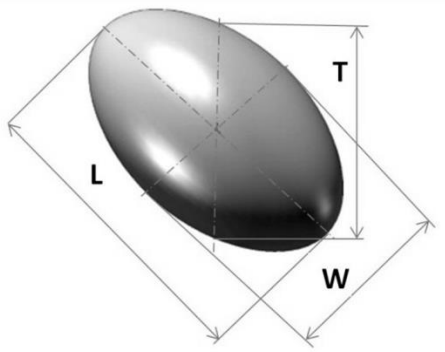


**Figure 3.** 3D ellipsoid model of Teff seed.

Areas of the seed in two perpendicular planes $S_I$, $S_{II}$ ($mm^2$) is calculated as area of ellipse (Eq. 6; Eq. 7)

$$S_I = \frac{\pi}{4} \cdot W \cdot L \quad (6)$$

$$S_{II} = \frac{\pi}{4} \cdot W \cdot T \quad (7)$$

where: L – axis length (mm); W – axis width (mm); T – axis thickness (mm).

Circumference of ellipse $O_I$ (mm) is given by Eq. 8 (Zill et al., 2011)

$$O_I = \frac{\pi}{2} \cdot \left[ \frac{3}{2} \cdot (W + L) - \sqrt{W \cdot L} \right] \tag{8}$$

Ratios of axis length φ (–), χ (–) are described by Eq. 8; Eq. 9.

$$\varphi = \frac{W}{L} \tag{9}$$

$$\chi = \frac{S_I}{S_{II}} = \frac{L}{T} \tag{10}$$

Using Eq. 8, Eq. 6 and Eq. 9 the formula for axis length calculation (Eq .11) were derived.

$$L = \frac{\frac{4}{3} \cdot \frac{O_I}{\pi} + \frac{2}{3} \cdot \sqrt{4 \cdot \frac{S_I}{\pi}}}{1 + \varphi} \tag{11}$$

And then using Eq.10 formula for axis thickness calculation (Eq. 12) was determined.

$$T = \frac{L}{\chi} \tag{12}$$

And then using Eq. 9 formula for axis width calculation (Eq. 13) was determined.

$$W = \varphi \cdot L \tag{13}$$

Volume of the ellipsoid was calculated by Eq. 14.

$$V_m = \frac{4}{3} \cdot \pi \cdot W \cdot T \cdot L \tag{14}$$

## RESULTS AND DISCUSSION

Measured dimensions, areas and circumferences of Teff seeds with their standard deviations are presented in Table 1 and histogram of Teff seed geometric diameters for seven groups of dimensions is shown in Fig. 4.

**Table 1.** Measured dimensions, areas and circumferences of Teff seed

| | $L^*$ (mm) | $W^*$ (mm) | $T^*$ (mm) | $S_I^*$ ($mm^2$) | $S_{II}^*$ ($mm^2$) | $O_I^*$ (mm) | $O_{II}^*$ (mm) |
|---|---|---|---|---|---|---|---|
| Amount | 1.191 | 0.632 | 0.608 | 0.663 | 0.360 | 3.227 | 2.025 |
| Standard deviation | 0.109 | 0.064 | 0.099 | 0.164 | 0.128 | 0.224 | 0.232 |

It is evident that Teff seeds have very small dimensions in comparison to the commonly used food grains (Bultosa & Taylor, 2004; Arendt & Zannini, 2013; Bultosa, 2016; Wrigley et al., 2016) which also correspond to the already published studies (Zewdu & Solomon, 2007; D'Silva et al., 2011; Gebremariam et al., 2014). From the sphericity (Table 2) it is also clear that the shape of the seeds are very similar to the shape of hempseeds (Sacilik et al., 2003) or flaxseeds (Coşkuner & Karababa, 2007) and linseeds (Selvi et al., 2006). From conducted experiments (Table 1) implies that when the seeds are compared dimensionally they are not similar seeds commonly used for food or industrial purposes (Arendt & Zannini, 2013; Bultosa, 2016).

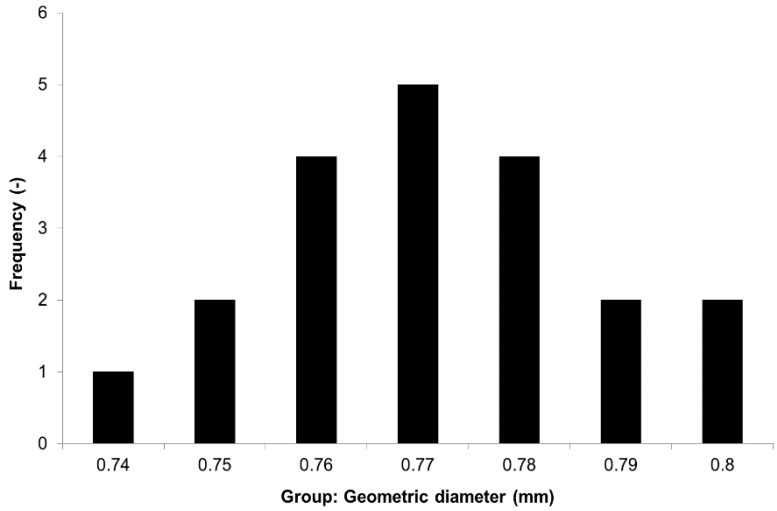


**Figure 4.** Histogram of Teff seed geometric diameters for seven groups of dimensions.

From measured amounts (Table 1) dimensions, sphericity, volumes of Teff seed's ball model were calculated and they are shown in Table 2.

**Table 2.** Calculated amounts of Teff seed's ball model

| $D_a$ (mm) | $D_g$ (mm) | $\phi$ (-) | $S_a$ ($mm^2$) | $S_g$ ($mm^2$) |
|---|---|---|---|---|
| 0.810 | 0.771 | 0.647 | 0.515 | 0.605 |

With aid of measured amounts (Table 1) dimensions, volumes, areas and circumferences of Teff seed's ellipsoid model were also determined and they are shown in Table 3.

**Table 3.** Calculated amounts of Teff seed's ellipsoid model

| L (mm) | W (mm) | T (mm) | $V_m$ ($mm^3$) | $S_I$ ($mm^2$) | $S_{II}$ ($mm^2$) | $O_I$ (mm) | $O_{II}$ (mm) |
|---|---|---|---|---|---|---|---|
| 1.295 | 0.687 | 0.703 | 2.621 | 0.697 | 0.302 | 3.181 | 1.948 |

From statistical analysis (Table 4) which was done by Student t test for level of significance 0.05 (*t-test*, $n = 20$, $P > 0.05$) it follows that values for ellipsoid model are statistically significant with measured amounts and that values of ball's model based on arithmetic diameter is statistically different from measured values. The area of ball model based on the geometric mean is statistically significant with measured area in plane I but, it is statistically different from the measured area in plane II. It is clear that the most suitable model is ellipsoid's model that dimensions, areas and circumferences adequately correspond to the true dimensions and shape of the Teff seeds.

**Table 4.** Statistical analysis measured values with models' values

| Compared amount | $S_a$ | $S_g$ | $S_I$ | $S_{II}$ | $O_I$ | $O_{II}$ |
|---|---|---|---|---|---|---|
| *T-test* value | 3.93 | 1.54 | 0.94 | 1.96 | 0.89 | 1.44 |
| Critical value | 2.09 | 2.09 | 2.09 | 2.09 | 2.09 | 2.09 |

The necessity of using ellipsoid model instead ball model for mathematical descriptions of mechanical behaviour have been already published by studies focused on the virtual modelling of the cereals such are Jatropha seeds (Petrů et al., 2012; 2014), rice kernel (Lizhang et al., 2013) or rice seeds (Zhan et al., 2013) and they confirm determined results of this study. It implies that the development of mathematical models based on ellipsoid's shape could be integral part of mathematical models, described behaviour of seeds or bulk seeds. From already conducted experiments (Mohsenin, 1970; Zewdu & Solomon, 2007) follow that dimensions of this model must be depended on the moisture content and temperature and this factor must be taken into account when a complex model will be created.

Determined, described and verified ellipsoid model can be used not only for description of Teff seeds but also for description of other cereals. It is evident that the ellipsoid model depends on the ratio of cereal dimensions and that this model in case of a certain ratio of axis length can be transform into ball's model.

## CONCLUSIONS

The dimensions, circumferences and areas in two perpendicular planes of Teff seeds (*Eragostis tef)* were measured.

The shape of the Teff seed was discussed and on this base new ellipsoid model for the seeds shape was derived.

Ellipsoid model was compared with measured values and also with determined values of commonly used ball model.

From statistically analysis implies that this model on significance level 0.05 is significantly identical with measured values of the Teff seeds and that it describe the shape of the seeds more precisely than ball model.

This determined model can help more accurately set up and developed accurate mathematical model for describing mechanical behaviour of individual seeds as well as bulk seeds.

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# Development of new pig carcasses classification formulas and changes in the lean meat content in Latvian pig population

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**Abstract.** Pig classification is based on objective estimation of the lean meat content of the carcasses. The European Union established a common framework for the classification of pig carcasses. Carcass classification serves as a quality development tool to encourage the breeding of animals, from which it is possible to get high quality carcasses for processors and consumers. It is a common practice to recalculate pig carcasses classification formulas and update existing classification methods (or develop new methods) after every five years.
The representative samples of 145 pig carcasses from all regions of Latvia were used for the dissection trial. The precisely dissected carcasses with the warm carcass weight 60–110 kg were selected according to fat thickness and gender of pigs (the sex ratio were 50% females and 50% castrated males). From the experimental data were developed new formulas for the four methods Intrascope (Optical Probe); Manual method (ZP); Pork Grader (PG200); Optigrade MCP. During sampling the average warm carcass weight was 89.31 kg. New coefficient was detected and formula was developed for calculation of carcass standard presentation in all cases if some of the carcass parts are missing; for the missing head 8.345, for the missing tail 0.072, for the missing forefeet 0.764, for the missing hind feet 1.558. The comparison between the currently used and new experimentally obtained formulas showed difference up to 1.86% in lean meat content. The results suggest high accuracy of new regression formulas, which fully meets requirements of EU legislation.

**Key words:** pigs, carcass classification, lean meat content, meat quality.

## INTRODUCTION

The domestic pig is an important livestock species and an important protein source worldwide (Larson et al., 2007). All abattoirs slaughtering more than 200 pigs a week are required to classify pig carcasses under the European Union (EU) rules. The basic functions of the grading scheme are making price quotations comparable throughout all EU member states, enable accurate monitoring of the market situation, enabling producers to be rewarded for producing carcasses according to market requirements, and establishing an average price for pig carcasses for reference price calculations. Pig classification is based on objective estimation of the lean meat content of the carcass. The EU established a common framework for the classification of pig carcasses. Carcass

classification serves as a quality development tool to encourage the breeding of animals, from which it is possible to get high quality carcasses for processors and consumers (Causeur et al., 2003; Šprysl et al., 2007). As a consequence, all member states had to carry out a new dissection trial and assess new classification methods (Brondum et al., 1998; Busk et al., 1999; Scholz et al., 2002; Collewet et al., 2005).

Pig carcases are graded at the time of weighing (within 45 minutes after slaughtering); according to their estimated lean meat content. An EU grade (SEUROP) can then be allocated to a carcase by using the lean meat percentage.

Quality of animals can be affected by various factors (such as genetics, feeding etc.). Based on the above mentioned, it is a common practice to recalculate pig carcasses classification formulas and update existing classification methods (or develop new methods) after every five years.

## MATERIALS AND METHODS

A calibration trial was carried out in November 2013. Subsequently, the data analysis was also carried out in accordance with the description in Protocol part I (Jansons et al., 2013). The work was executed in the licensed slaughterhouse ZS 'Kumelītes' (approval number A006677).

Precisely splitted carcasses with the warm carcass weight of 60–110 kg were selected for the trial. Data collection was performed on 145 carcases by means of four instruments. The criteria of selection of sample population were fat thickness (as the reference method used with Intrascope) and sex of the pigs. The representative samples of 145 pig carcasses (Commission Regulation No 1249/2008) were used for the dissection trial. The pigs were collected by transport to the slaughterhouse from largest farms of four regions of Latvia (Vidzeme, Kurzeme, Zemgale, Latgale).

Based on the simplified detailed slaughter dissections, the lean meat content (Y) were established according to the approved formula in accordance with Commission Regulation (EC) No 1249/2008:

$$Y = 0.89 \times 100 \times \frac{TL + LM}{TL + DC}, \quad (1)$$

where *TL* – weight of tender loin, kg; *LM* – weight of lean meat in the shoulder, loin, ham and belly, kg; *DC* – weight of dissected cuts, kg.

To determine lean meat percentage in the carcass, measurements with Intrascope (Optical Probe) were carried out by inserting the device at 6 cm of the splitted carcass midline from the last rib.

The back fat thickness was determined by measuring with Intrascope. According to this index, the corresponding leanness class was established for the carcasses.

To determine lean meat percentage in the carcass with Manual ZP method, two manual measurements with caliper or electronic caliper were carried out:

muscle thickness measurement – M (distance between the cranial end of the *Gluteus medius* muscle and the edge of the *canalis vertebralis*);

backfat thickness measurement – G (the narrowest place over the *Gluteusmedius* muscle).

For the measurements, appropriate easy-to-use metal ruler or electronic caliper and data matrix were used.

Carcass lean meat percentage were calculated based on the measurement points.

The manual method ZP, should only be authorised in slaughterhouses having the limit of slaughters of not more than 200 pigs per week and using an electronic data input method with the limit of slaughters of not more than 500 pigs per week.

Electronic data input method is advanced Manual ZP method by using the electronic caliper. Caliper is fitted to computer via Blue-tooth connection. Lean meat content was calculated when input measured G and M in special slaughterhouse computer program. Caliper have no any wire. When first dimension (G) is measured operator must push measures key one time. Second dimension (M) could be measured same – pushing on measure key second time. After receiving of data (G, M) program make calculations and automatic classification in SEUROP classes, data also is writing in program data base automatically. For preventing of mistakes caliper shows measured fat depth and thickness of the muscle on led screen. This method was accurate and increases the speed of carcasses classification.

'Pork Grader 200', optical-electronic measurements were carried out. The measurements were taken from the last rib; puncture injection was within limits of $7 \pm 1$ cm, puncture output was $4 \pm 0.5$ cm from the carcass midline, perpendicularly to the carcass.

The Pork Grader 200 device consists of a measuring probe fitted to the gun-shaped case, data sheet printer and gage – test block. The pistol contains calculation software. At the end of the probe, 8–9 mm wide blade is placed, as well as a led-light with an adjacent light receiver (photodetector). Muscle tissues and fat tissues reflect different light wavelengths to the light indicator. Puncture of the measuring probe shall pass through the carcass by injecting it from the last rib at 7 cm and outputting it at 4 cm from the carcass midline. Probe withdrawing give the result in mm-s, which accuracy is $\pm$ 0.5 mm. The measurement indicates muscle tissue and fat tissue thickness with skin thickness. The measured values were converted into lean meat percentage by the help of a calculation program.

To determine lean meat percentage in the carcass, measurements with Optigrade MCP device were carried out. The puncture was injected between the last rib and the second to last rib; puncture injection was within limits of $7 \pm 1$ cm, puncture output was $4 \pm 0.5$ cm from the carcass midline, perpendicularly to the carcass.

The operation of the Optigrade MCP device was similar to the operation of the PG 200 (Pork Grader). It shows measurements of muscle tissue thickness and fat tissue thickness including skin thickness. The measured values were converted into lean meat percentage by the use of a calculation program.

**Statistical analysis.** Estimation of regression parameters for the instruments used in measurements were obtained with ordinary least square (OLS) regression by use of R programming tools (R version 2.14.0, The R Foundation for Statistical Computing). In total, data of 145 pig carcases were used in calculations. Root mean square error of prediction (RMSEP) was calculated by using leave-one-out approach (RMSEP(loot)) and k-fold cross-validation (RMSEP(mc)) approach. Cross-validation was performed by Monte Carlo method, randomly splitting the dataset into Training set including 1/3 of the data (37 observations), and Test set (108 observations). RMSEP(mc) was calculated as a mean from 1,000 iterations.

## RESULTS AND DISCUSSION

The distribution of pigs in the Latvia represents the breed and crossbreeds (Large White, Landrace, Large White x Landrace, Pietrain Hampshire, Duroc which are approved for meat production.

The carcases corresponded to the standard presentation in accordance with EU legislation, without bristles, hooves, thoracic, abdominal, and pelvic viscera, genital organs, diaphragm and flare fat, with head, feet and tail.

The carcasses were selected according to gender: the sex ratio was 50% females and 50% castrated males (73 females and 72 castrated males).

The average warm carcass weight was 89.31 kg and fat thickness with Intrascope 17.88 mm (Table 1). In most European countries slaughter pigs are brought to slaughterhouses at the age of six months, which means that their carcass weight is in the range of 75–90 kg and the average lean meat percentage ranges from 55 to 60% (Šprysl et al., 2007). The share of pigs with extremely low or high carcass weight represents only 1.3% of all the pigs slaughtered. The average pig carcass weight in Czech Republic was 87.2 kg (Kvapilk et al., 2009), which is higher than in Slovenia – 81 to 83 kg (Čandek-Potokar et al., 2004) and comparable with Netherlands – 86.2 kg (Van Wijk et al., 2005).

**Table 1.** Results of weighing and measurement of fat thickness of the carcasses, n 145

| Characteristics | Mean | Standard Deviation | Minimum | Maximum |
|---|---|---|---|---|
| Carcass weight (warm), kg | 89.31 | 8.90 | 58.72 | 110.00 |
| Carcass without the head (warm), kg | 81.87 | 8.25 | 53.17 | 103.16 |
| Carcass left side (cold), kg | 39.91 | 8.25 | 25.67 | 50.25 |
| Fat thickness (with Intrascope), mm | 17.58 | 3.43 | 11.00 | 27.00 |

In the trial new formulas for four methods were calculated, which are approved by the European Commission and can be used for the Latvian pig population in order to carry out the carcasses grading. Relationship between residuals, lean meat percentage predicted and dissected shown in (Fig. 1, 2).

**Intrascope** (Figs 1, 2a)

The lean meat content ($\hat{Y}$) in pig carcass can be predicted by Intrascope based on the equation (N = 145; RMSEP(loot) = 1.968; RMSEP(mc) = 1.967):

$$\hat{Y} = 66.6708 - 0.3493 \times F \quad (2)$$

where $F$ – fat thickness.

**Manual ZP method** (Figs 1b, 2b)

The lean meat content ($\hat{Y}$) in pig carcass can be predicted by ZP method based on the equation (N = 145; RMSEP(loot) = 2.001; RMSEP(mc) = 2.000):

$$\hat{Y} = 60.5214 - 0.2579 \times G + 0.0525 \times M \quad (3)$$

where $G$ – fat thickness, $M$ – thickness of muscle.

**Pork Grader 200** (Figs 1c, 2c)

The lean meat content ($\hat{Y}$) in pig carcass can be predicted by Pork Grader 200 based on the equation (N = 145; RMSEP(loot) = 1.839; RMSEP(mc) = 1.841):

$$\hat{Y} = 64.4502 - 0.4364 \times F + 0.0381 \times M \quad (4)$$

where $F$ – fat thickness, $M$ – muscle thickness.

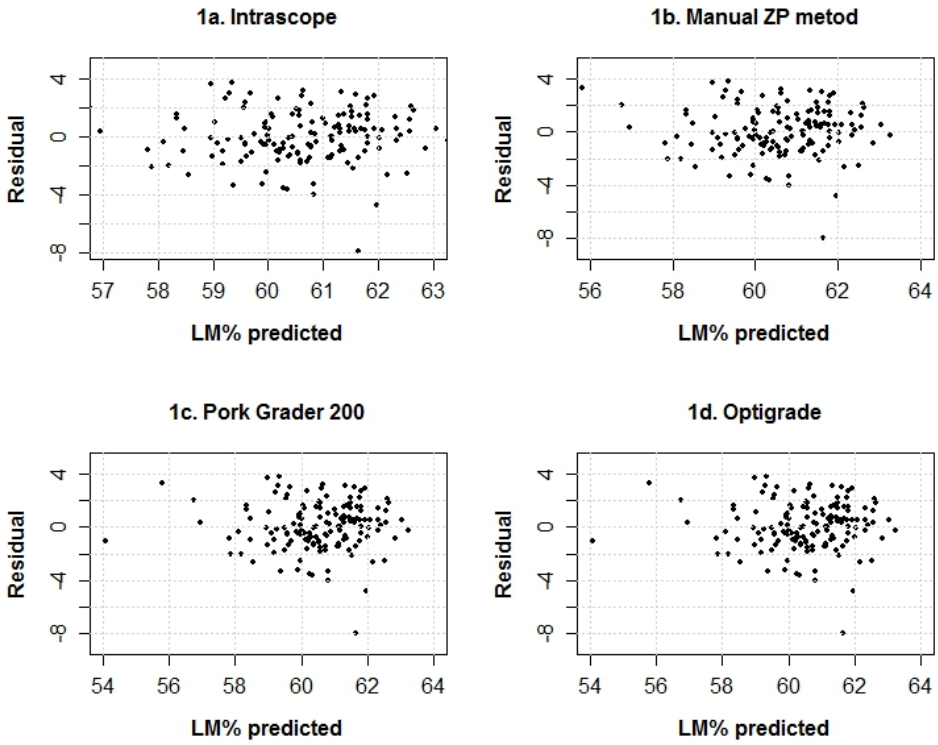


**Figure 1.** Relationship between the predicted lean meat percentage (LM%) and the residuals.

**Optigrade MCP** (Figs 1d, 2d)

The lean meat content ($\hat{Y}$) in pig carcass can be predicted by Optigrade MCP based on the equation (N = 145; RMSEP(loot) = 1.841; RMSEP(mc) = 1.842):

$$\hat{Y} = 66.7787 - 0.4464 \times F + 0.0018 \times M \quad (5)$$

where $F$ – fat thickness, $M$ – muscle thickness.

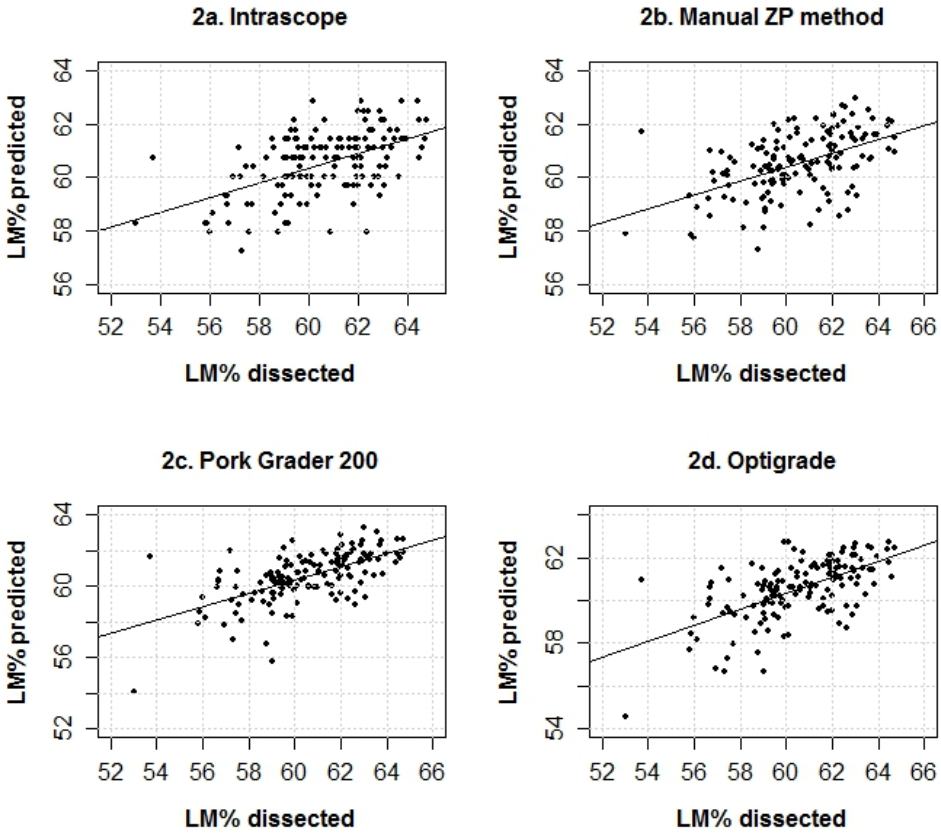


**Figure 2.** Relationship between the dissected and predicted lean meat percentages (LM%).

While testing different devices on carcasses in Poland in 2011, following evaluation errors RMSEP were reported: UltraFom 300 – 2.07%, IM-03 – 1.89%, CGM – 2.16%, Fat-o-Meat'er II – 2.18% (Lisiak et al., 2012; Lisiak et al., 2015). These evaluation errors were lower than with the ZP method (2.33%) obtained in current research. Research carried out by other researchers has also confirmed this: Engel et al. (2012) found the following RMSEP errors of different devices: HGP7 – 2.10% and CGM – 2.20%. Font-i-Furnols & Gispert (2009) proved a 1.8% RMSEP error for the Fat-o-Meat'er, 2.3% for UltraFom 300, 1.9% for AutoFom and 2.3% for VCS 2000. RMSEP error values determined for the ZP method were slightly greater reaching 2.52% in Germany, 2.38% in Sweden, 2.45% for gilts and 2.49% for castrated males in France (Daumas & Dhorne, 1998). Regardless of the differences found in RMSEP values, all methods in Latvia represents a legal and acceptable RMSEP value below 2.5% that is within the EU allowed limit.

Standard carcass presentation is required under EU rules. There are slaughterhouses in Latvia which separate carcass parts during pig carcass processing, therefore it is necessary to determine quotients of these parts. The following fixed coefficients were calculated if some of the following carcass parts are missing and shall be applied in all cases (Table 2).

**Table 2.** Index of the separated carcass parts, n 145

| Cuts | Mean, kg | Standard deviation | Minimum, kg | Maximum, kg | Quotient |
|---|---|---|---|---|---|
| Head | 7.446 | 0.865 | 5.460 | 9.620 | 8.345 |
| Tail | 0.069 | 0.063 | 0.040 | 0.800 | 0.072 |
| Forefeet | 0.677 | 0.034 | 0.601 | 0.736 | 0.764 |
| Hind feet | 1.387 | 0.109 | 1.059 | 1.595 | 1.558 |

Notwithstanding the standard presentation laid down in point B. III of Annex IV to Regulation (EU) No 1308/2013, pig carcasses in Latvia may be presented without the head, the tail, the forefeet and/or the hind feet before being weighed and graded.

In order to establish quotations for pig carcasses on a comparable basis, the following fixed coefficients shall be applied in all cases if some of the following carcass parts are missing:

- for the missing head 8.345
- for the missing tail 0.072
- for the missing forefeet 0.764
- for the missing hind feet 1.558

The standard presentation carcass weight shall be calculated according to the formula:

$$SPCW = \frac{100 \times W}{(100 - C)}, \quad (6)$$

where: $SPCW$ – standard presentation carcass weight; $W$ – carcass weight (without missing part(s)); $C$ – coefficient(s) for missing part(s)).

The comparison between the currently used and in the new formula for prediction of lean meat percentage showed difference up to 1.856% in average (Table 3).

**Table 3.** Comparision of the lean meat content with old and new formulas

| Traits | ZP | Intrascope | Pork Grader 200 | Optigrade |
|---|---|---|---|---|
| Lean meat by old formula, % | 59.99 | 58.67 | 59.51 | 59.43 |
| Lean meat by new formula, % | 60.53 | 60.53 | 60.53 | 60.52 |
| Difference, % | **0.532** | **1.856** | **1.020** | **1.094** |
| Lean meat dissected, % | 60.53 | 60.53 | 60.53 | 60.53 |
| Lean meat by old formula % | 59.99 | 58.67 | 59.51 | 59.44 |
| Difference, % | **0.532** | **1.857** | **1.0167** | **1.096** |
| Lean meat dissected,% | 60.53 | 60.53 | 60.53 | 60.53 |
| Lean meat by new formula, % | 60.53 | 60.53 | 60.53 | 60.53 |
| Difference, % | **0.001** | **0.000** | **-0.003** | **0.002** |

Differences in lean meat content between dissected carcasses and currently used old formulas were up to 1.857% on average per carcase. In contrast, the comparison with the new formula, difference does not exceed 0.003%. Consequently, we found that the lean meat percentage content in carcasses for pig population in the Latvia changed slightly. Various factors such as genetics, feeding, etc. affected the changes in quality of

pig herds, results of our research showed lean meat content in carcasses of the Latvia pig population has decreased.

## CONCLUSIONS

Trial for four methods (Intrascope (Optical Probe); manual method (ZP); Pork Grader (PG 200); Optigrade MCP) to estimate formulas for grading pig carcasses were developed. The comparison between the currently used and in the new formula for prediction of lean meat percentage showed difference up to 1.856% in average These methods and new formula have been approved in the EU and allowed to use in Latvia.

New coefficient was detected and formula was developed for calculation of carcass standard presentation in all cases if some of the carcass parts are missing; for the missing head 8.345, for the missing tail 0.072, for the missing forefeet 0.764, for the missing hind feet 1.558. Differences in lean meat content between dissected carcasses and the new formula does not exceed 0.003%. The results suggest high accuracy of new regression formulas, which fully meets requirements of EU legislation. They represent good presumption for more objective pig grading and equitable payment for pig producers in the Latvia.

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# Effect of lovage phenolics to formation of acrylamide in French fries

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**Abstract.** One of the novel methods for reduction of acrylamide in food is application of plant phenolics in technological process of Latvian plants as lovage contain significant amounts of plant phenolics and other natural antioxidants. The aim of current research was to determine the effect of lovage extracts to the formation of acrylamide in French fries. Variety 'Lenora' potatoes were used. Potatoes were sliced and blanched in hot water (85 ± 2 °C 8 min). After blanching samples were treated with lovage water and ethanol extracts and four samples were obtained: control (without additional treatment); SW – sprayed with water extract, IMW – immersed in water extract, SE sprayed with ethanol extract. After treatment all samples were fried in oil (180 ± 2 °C) for 7 minutes. Total phenolic (TPC), vitamin C content and antioxidant activity (DPPH and ABTS) were determined for all samples before and after frying. For fried potatoes acrylamide and breaking force with texture analyser were determined. TPC of samples during frying decreased significantly but comparing fried samples the highest TPC in SE sample was determined. The highest DPPH radical scavenging activity was observed in samples treated with water extract but during frying the DPPH activity for all treated samples was lower than to control sample. The most significant changes in ABTS radical scavenging activity were observed and also the highest activity of sample SE was observed. Vitamin C content decreased significantly during frying, the highest vitamin C content in SE sample was determined. The highest maximal breaking force of fried potatoes was detected for sample IMW, but the lowest for sample SE. The lowest acrylamide content was found in sample, which was sprayed with lovage-water extract.

**Key words:** French fries, lovage, treatment, ABTS, ascorbic acid, breaking force.

## INTRODUCTION

Potatoes are the fourth-most-consumed food crop in the world, after rice, wheat, and corn. The most popular potato products are French fries, chips, and dried potato products. Frozen French fries are the top U.S. potato product export, accounting for more than half of total potato export volume. In 2009, exports of frozen French fries totalled 3.0 billion pounds (fresh-weight-equivalent basis), valued at $635 million. Canada is the largest supplier, followed distantly by Mexico, the Netherlands, and Germany (USDA, 2014).

Potato products such as French fries and chips contains high levels of acrylamide that forms during thermal treatment in reaction between sugars and asparagine.

Acrylamide is a substance that is produced naturally in foods as a result of high-temperature cooking, e.g., baking, grilling, or frying (Mottram et al., 2002).Average daily intake levels of this Maillard reaction product are estimated to be 0.3–0.7 μg acrylamide $kg^{-1}$ $day^{-1}$ (Dybing et al., 2005).

Potatoes are rich in both asparagine and glucose. Asparagine is the free amino acid presented in high amounts in potatoes (93.9 mg 100 $g^{-1}$) (Martin & Ames, 2001).

Due to urotoxic, genotoxic and probable carcinogenic properties of acrylamide several methods are developed to reduce its content in different food matrixes such as prevention of reducing sugar liberation during the storage period of food materials (Fiselier & Grob, 2005), change of heat processing methods (Granda et al., 2004), selection of suitable cultivar and storage temperature of food materials (Grob et al., 2003; Rommens et al., 2008), modification of pH (Jung et al., 2003) reduction of ammonium bicarbonate (Levine & Smith, 2005), fermentation (Baardseth et al., 2006), addition of amino acids like glycine (Bråthen et al., 2005), etc. As Rommens et al. (2008) reported an eventual replacement of existing potatoes by low-asparagine varieties would lower the ingestion of acrylamide by approximately 30% but still some acrylamide can form in the processed products. In the investigations of Pedreschi et al. (2007) potato strip immersion in citric acid solution of 10 $gL^{-1}$ reduced the acrylamide formation during frying more than the strip immersion in sodium pyrophosphate solution of 10 $gL^{-1}$ (53% vs. 17%, respectively – average values for the three temperatures tested). Acrylamide formation decreased dramatically as the frying temperature decreased from 190 to 150 °C for all the pre-treatments tested (Pedreschi et al., 2007).

One of the novel methods for reducing of acrylamide content in final products is application of plant phenolics in technological process. As Yanbing et al. (2015) are summarising, plant polyphenols, one part of phytochemicals, have attracted a great deal of attention for its natural antioxidative feature. After the discovery of acrylamide in food, plant polyphenols were applied to inhibit acrylamide formation. The influence of polyphenols was depended on the structure, concentrations, and antioxidant capacity, as well as reaction conditions, but also some other mechanisms were involved (Yanbing et al., 2015). In the study of Zhang et al. (2007) potato crisps and French fries were immersed into different contents of antioxidant of bamboo leaves (AOB) solution, and the frying processing parameters were optimized. The results of this research showed that nearly 74.1% and 76.1% of acrylamide in potato crisps and French fries was reduced when the AOB addition ratio was 0.1% and 0.01% (w/w), respectively. The maximum inhibitory rate was achieved when the immersion time was designed as 60 s. Zhang & Zhang (2007) investigated the efficiency of antioxidant of bamboo leaves (AOB) and extract of green tea (EGT) on the reduction of acrylamide in fried bread sticks and summarized the optimal levels of two additives. Results showed that nearly 82.9% and 72.5% of acrylamide were reduced when the addition levels of AOB and EGT were 1 and 0.1 g $kg^{-1}$, respectively. The study indicated that both AOB and EGT could significantly reduce the acrylamide content generated in fried bread sticks and keep original flavour and crispness of fried bread sticks (Zhang & Zhang, 2007). The addition of rosemary extract to the frying oil significantly ($P < 0.05$) reduced the acrylamide content by up to 38%. The significantly smaller change in acrylamide concentration in the potato deep-fried in oil with the addition of rosemary extract compared to potato deep-fried in oil with the addition of butylatedhydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ), and the tocopherols ($P < 0.05$) showed that this rosemary

extract has exceptional carry-over protective effects for deep-fried food, because of its high heat stability (Urbančič et al., 2014).

Many aromatic plants contain compounds that can act as natural antioxidants (Raghavan, 2000). The antioxidant characteristics of plant derived materials can be attributed to their polyphenols. Many plants of *Umbelliferae* family also contain several bioactive phytochemicals such as flavonoid and coumarins, which are reported to have curative, preventive, or nutritive value (Cherng et al., 2008).

Latvian plants lovages are characteristic for our region, but they are rarely used as food ingredient in nowadays. Lovages already from ancient times have been used as herbal plant (Raghavan, 2000). They contain relevant amounts of plant phenolics and other natural antioxidants. Lovage (*Levisticumofficinale* L.) is a perennial herb belonging to the *Umbelliferae* family, with a characteristic earthy, celery-like flavour and smell (Szebeni-Galambosi et al., 1992). All parts of the plant being strongly aromatic with a characteristic earthy, celery-like flavour and smell (Szebeni-Galambosi et al., 1992; Raghavan, 2000). Seeds, leaves and roots of lovage (fresh, powdered and as essential oils), are commonly used in Europe for flavouring foods and beverages and for their medicinal properties (Cu et al., 1990). Lovage root has also been known for centuries as a medicine possessing spasmolytic, diuretic and carminative activities (Raghavan, 2000).

Lovage bioactivity is the basis for the selection of this plant for investigations of possible reduction of acrylamide and other quality changes in fried products. Therefore the aim of the current research was to determine the effect of lovage extracts on the formation of acrylamide in French fries.

## MATERIALS AND METHODS

### Raw materials

Fresh lovage (*Levisticumofficinale* L.) leaves were collected in Latvia in June 2015 and frozen (-20 ± 2 °C) immediately after collection.

Potatoes 'Lenora' were grown in experimental fields of State Priekuli Plant Breeding institute in 2015. For experiments potatoes were washed, peeled and sliced into strips (0.8 × 0.8 × 6 cm) by longest distance.

### Extraction procedure of phenolic compounds from lovage

For extraction of phenolic compounds from lovage as solvents water and ethanol were used. According to the previous investigations (Tomsone, 2015) the lovage leaves were chosen as the plant material for extract using ultrasonic facilitated extraction. Two extracts were used. Both extracts were prepared using ultrasound assisted extraction (UAE) where the output power was 250 W and the frequency was 50 kHz.

The extract with ethanol was made at room temperature +21 ± 2 °C, but the water extracts were prepared at +60 °C temperature for better extraction.

The first extract was water extract of lovage leaves. Frozen lovage leaves (95 g) were homogenized and placed into conical flask in which 500 mL water were added. Then the flask was sonicated one hour in an ultrasonic water bath at 60 °C temperature for better extrction. Extracts were cooled till room temperature and then filtered (cellulose filter paper No.89).

The second extract was 95% ethanol extract of lovage leaves. Frozen lovage leaves (95 g) were homogenization and placed into conical flask in which 1 L ethanol wasadded. Then the flask was sonicated one hour in an ultrasonic water bath at ambient temperature. Extracts were filtered (filter paper No.89).

The extraction process was performed using single extraction process (without re-extracting) and repeated in triplicate. For extracts total phenol content and radical scavenging activity was determined (in triplicate for each extraction).

**French fries preparation technology**

Potatoes were divided into 12 batches (four samples in three replications) and each was treated separately. Potato samples were first blanched in hot water (85 °C 8 min) and subjected to analysis. After blanching samples were sprayed or immersed in lovage-water extract or sprayed with ethanol extract (Table 1). After spraying with lovage extracts and immersion in water extract the sample were left for 10 min at room temperature (+20 ± 1 °C) before frying.

**Table 1.** The abbreviations of the samples used in experiments

| Sample codes | Explanation | Extract ratio (g $g^{-1}$), treatment time (min) |
|---|---|---|
| C_B | control, blanched | without additional treatment |
| SW_B | sprayed with water extract, blanched | ratio extract/potatoes=11/200, 10 |
| IMW_B | immersed in water extract, blanched | ratio extract/potatoes=190/200, 10 |
| SE_B | sprayed with ethanol extract, blanched | ratio extract/potatoes=11/200, 10 |
| C_F | control, fried | |
| SW_F | sprayed with water extract, fried | |
| IMW_F | immersed in water extract, fried | |
| SE_F | sprayed with ethanol extract, fried | |

After treatment all samples were fried in oil (180 ± 2 °C) for 7 minutes (200 g potatoes in 1l of oil).Rapeseed oil 'Oleina' produced in Kruszwica, Poland was used for frying. For further chemical analyses average sample combining French fries from three replications were used.

**Physical analysis**

For lovage leaves and potatoes samples the moisture content was determined according to the standard ISO 6496:1999 and all results were expressed on dry basis. pH was measured by JENWAY 3510 pH-meter, standard method LVS ISO 5542:2010.

*Texture analyses*

The textural properties of potato straws were measured in terms of the cutting force. A texture Analyzer TA.HDplus (Stable Microsystems, UK) was used for cutting force determination. Potato straws were cut using blade set with knife (HDP/BSK), moving at a pre-test speed 1.00 mm $s^{-1}$, and test speed of 10 mm $s^{-1}$ over a distance of 15.0 mm. The numerical results were expressed in N.

**Chemical analysis**

*Extraction of phenolic compounds from potatoes*

The homogenized samples were extracted with ethanol (80/20 w/w) in a conical flask with a magnetic stirrer (magnet 4.0 × 0.5 cm) at 700 rpm for 1 h at room temperature (20 ± 1 °C). The extracts were then filtered (paper No.89). The extraction process was done in triplicate.

*Determination of total phenolic compounds*

The total phenolic content (TPC) of the lovage and potato extracts was determined according to the Folin-Ciocalteu spectrophotometric method (Singleton et al., 1999) with some modifications. The absorbance was measured at 765 nm and total phenols were expressed as the gallic acid equivalents (GAE) 100 $g^{-1}$ dry weight (DW) of plant material.

*Determination of total flavonoid compounds*

The total flavonoid content (TFC) was measured by a spectrophotometric method (Kim et al., 2003). To 0.5 mL of extract 2 mL of double distilled H2O was added, and mixed with 0.15 mL of 5% sodium nitrite (NaNO2) (50 g $L^{-1}$). After 5 min, 0.15 mL of 10% aluminium chloride (AlCl3*6H2O) solution was added. The mixture was allowed to stand for another 5 min, and then 1 mL of the 1M sodium hydroxide (NaOH) was added. The reaction solution was mixed well. After 15 min of incubation at room temperature, the absorbance was measured at 415 nm.

The absorbance was measured at 415 nm and total flavonoids were expressed as catechin equivalents (CE) 100 $g^{-1}$ DW of the lovage.

*Determination of antioxidant activity*

Antioxidant activity of the plant extracts was measured on the basis of scavenging activities of the stable 2,2-diphenyl-1-picrylhydraziyl (DPPH˙) radical as outlined by Yu et al. (2003). The absorbance was measured at 517 nm. The radical scavenging activity (RSA) of extract was also measured by 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic) acid (ABTS˙$^{+}$) radical cation assay (Re et al., 1999). For the assessment of extracts, the ABTS˙$^{+}$ solution was diluted with a phosphatebuffersolution to obtain the absorbance of 0.800 ± 0.030 at 734 nm. The RSA was expressed as TE 100 $g^{-1}$ DW of plant material. The higher the Trolox equivalent antioxidant capacity (TEAC) of a sample, the stronger the antioxidant activity.

*Determination of vitamin C*

The content of ascorbic acid was determined by titration with 0.05-M iodine solution (Jansons, 2006; Kampuse et al., 2014). The French fries samples (12.5 g) were poured with 50 mL6% solution of oxalic acid and homogenized. Then the sample was filtered. 2 mL of 1% solution of starch was added to 10 mL of filtrate and the filtrate was titrated until the colour changed which did not disappear during a 30 sec interval. For standard solution of ascorbic acid 20 mg of ascorbic acid were dissolved in 100 mL of the oxalic acid solution. Two mL of the starch solution was added to 25 mL of the standard-solution and the mixture was titrated. The content of vitamin C (ascorbic acid) mg per 100 g of the product dry matter was calculated using the following equation (1):

$$C = 5{,}000 \cdot \frac{V_{sample}}{m \cdot V_{standard}}, \quad (1)$$

where: $V_{sample}$ – volume of the iodine solution titrated in a sample, mL; $V_{standard}$ – volume of the iodine solution titrated in a standard solution, mL; m – the weight of a sample, g.

*Determination of acrylamide content*

The content of acrylamide in samples was detected in certified laboratory of Food safety, animal health, and environment scientific institute BIOR using High performance liquid chromatography method (HPLC) Acquity UPLC in combination with mass spectrometer QTRAP 5500.

Sample (1 g), the internal standard (500 ng $g^{-1}$) and 5 mL of hexane were added into a 50 mL centrifuge tube, then the tube was vortexed. Distilled water (10 mL) and acetonitrile (10 mL) were added followed by the the QuEChERS extraction salt mixture (4 g anhydrous MgSO4 and 0.5 g NaCl). The sample tube was shaken for 1 min vigorously and centrifuged at 4,500 g for 5 min. The hexane layer was discarded, and 1mL of the acetonitrile extract was transferred to a tube containing 50 mg of PSA-sorbent and 150 mg of anhydrous MgSO4. The tubes were vortexed for 30 s and then analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The quantitative analysis of acrylamide was based on the pre-treatment of selected final products and performed by LC-MS/MS using a Waters Acquity coupled to a 5500 QTrap mass spectrometer (AB Sciex). The separation of acrylamide was achieved with Luna 3 um HILIC column (50 x 2.00 mm i.d., 3 mm; Phenomenex). Methanol (6%) in acidified acetonitrile (0.1% formic acid) was used as a mobile phase (flow rate 0.3 mL $min^{-1}$, column temperature 40 °C and injection volume 10 mL). The detection by MS/MS was performed using electrospray ionization in the positive mode. The MRM transitions were m/z 72.0 → 54.9 and 72.0 → 44 for acrylamide and 75.0 → 58.0 for acrylamide-d3. The limit of quantification for acrylamide was 50 mkg $kg^{-1}$.

**Statistical analysis**

Experimental results aremeans of three replications and were analyzed by Microsoft Excel 2010 and SPSS 17.00. Analysis of variance (ANOVA) and Tukey's test were used to determine differences among samples. Differences were considered as significant at $p < 0.05$.

## RESULTS AND DISCUSSION

**Total phenol content and radical scavenging activity in lovage extracts**

Extraction of phenolic compounds from lovage was performed using two solvents – water and ethanol. The recovery of polyphenols from plant materials is influenced by the plant matrix, method, solubility in the solvent used for the extraction process (Zhou & Yu, 2004; Spigno et al., 2007; Nićiforović et al., 2010; Michiels et al., 2012). Solvent polarity plays a key role in increasing phenolic solubility (Grigonis et al., 2005; Naczk & Shahidi, 2006; Michiels et al., 2012). The properties of extracting solvents significantly affected the measured total phenolics content (± 25% variation) and antioxidant capacity (up to 30% variation) in fruits and vegetables (Michiels et al., 2012). Solvents, such as methanol, ethanol, acetone, propanol and ethyl acetate have been

commonly used for the extraction of phenolics from fresh product (Durling et al., 2007; Alothman et al., 2009). Due to toxicity, from these solvents only ethanol was selected. Several authors reported ethanol as the best solvent for recovery of polythenols (Grigonis et al., 2005; Tomsone et al., 2012; Tomsone & Krūma, 2013). Although efficiency of water as solvent for phenolic compounds recovery lower (Medouni-Adrar et al., 2015), it was selected due to safety, cost reasons and fits with green extraction approach.

The highest total phenol content (TPC), total flavonoid content (TFC), and also free radical scavenging activity (DPPH) was detected in ethanol extract (Table 1). Results of TPC are lower compared with those obtained by Tomsone et al. (2015) reporting up to 2,205 mg GAE 100 $g^{-1}$ TPC in ethanol extract obtained by Soxlet extract and 1,573 mg GAE 100 $g^{-1}$ TPC in ethanol extract obtained by accelerated solvent extraction.

Similar results also were found in experiments about other plants – leaves of *Moringaoleifera* L., where TFC increased by increasing concentration of ethanol in extraction (Vongsak et al., 2013).But the highest cationic binding capacity (ABTS) was obtained in lovage-water extract (Table 2).

**Table 2.** Total phenol content (TPC), total flavonoid content (TFC) andantiradicalactivity in lovage extracts depending on solvent

| Extract | TPC, mg GAE 100 $g^{-1}$ | TFC, mg CE 100 $g^{-1}$ | DPPH, mM TE100 $g^{-1}$ | ABTS, mM TE 100 $g^{-1}$ |
|---|---|---|---|---|
| 2% lovage water extract | $819.01 \pm 4.92^{b}$ | $1{,}210.87 \pm 13.88^{b}$ | $52.4 \pm 0.45^{b}$ | **55.73** $\pm 2.32^{a}$ |
| 1% lovage ethanol extract | **1,034.08** $\pm 9.55^{a}$ | **1,619.93** $\pm 14.58^{a}$ | **97.44** $\pm 3.47^{a}$ | $50.29 \pm 0.31^{b}$ |

Extraction is strongly influenced by plant matrix because in the study on *Artemisiaargyi* L. leaves TPC in methanolic extract was higher comparing to aqueous extract, although *Pyrrosialingua* L. leaves showed opposite effect (Cai et al., 2004). Literature data showed that ethanol was the best solvent for extraction of bioactive substances from horseradish roots and leaves (Tomsone et al., 2012; Tomsone & Kruma 2013).

**Potato moisture content and pH value**

During frying the moisture content and also pH value significantly decreased in all evaluated samples. The highest moisture content after frying was in the sample treated with ethanol extract – it was significantly higher than in the samples treated with water (Table 3).

**Table 3.** The moisture content and pH value in blanched potatoes and French fries treated with different extracts

| Sample | Moisture, % | pH |
|---|---|---|
| C_B | $70.82 \pm 0.26^{b*}$ | $6.17 \pm 0.09^{a}$ |
| SW_B | $73.13 \pm 0.02^{a}$ | $5.76 \pm 0.13^{b}$ |
| IMW_B | $72.94 \pm 0.18^{a}$ | $5.83 \pm 0.18^{b}$ |
| SE_B | $70.68 \pm 0.08^{b}$ | $5.86 \pm 0.15^{b}$ |
| C_F | $42.76 \pm 0.84^{d}$ | $5.51 \pm 0.1^{c}$ |
| SW_F | $41.58 \pm 0.64^{e}$ | $5.64 \pm 0.07^{bc}$ |
| IMW_F | $40.93 \pm 0.61^{d}$ | $5.58 \pm 0.06^{c}$ |
| SE_F | $44.43 \pm 1.02^{c}$ | $5.69 \pm 0.05^{bc}$ |

*****Values, marked with the same letter, are not significantly different at $P < 0.05$.

According to Cheng et al. (2013) water content may affect the distribution and the status of the antioxidant applied, as well as its combination and reaction with acrylamide or its intermediates. There were no significant differences in pH value between processed potatoes after blanching, and also in deep-fried potatoes this parameter did not differ significantly. Frying only a slightly decreased pH value (Table 3). According to the data mentioned in literature, the pH values between 7.0 and 8.0 are the most suitable ones for acrylamide formation. Therefore, the pH value can be adjusted to a lower value to reduce the formation of acrylamide in industrial applications (Weisshaar, 2004; Mestdagh et al., 2008; Cheng et al., 2013). Relatively low pH values (5.51–5.69) in fried potatoes of our experiment could result in lower acrylamide content in the end product.

**TPC**

After treatment with water extracts the total phenol content of the samples did not increase significantly ($P > 0.05$), but after frying with ethanol sprayed fries had significantly ($P < 0.05$) higher phenol content. During frying the highest TPC loss (33.92%) was detected in an aqueous extract soaked fries, but the lowest - with ethanol sprayed fries (24.27%).

**DPPH**

The highest free radical binding capacity similarly as to TPC was in blanched and in lovage-water extract soaked samples although the differences between treated samples were not significant. But after frying the DPPH activity for all treated samples were even lower than in a control sample, and the lowest it was in a sample soaked in lovage-water extract – the decrease of DPPH was 52.41%.

**ABTS**

The cationic binding capacity (ABTS) after frying did not significantly change for control sample, but it was decreased in the samples treated with water extract (for 10.74–13.42%).For the samples treated with ethanol extract the ABTS activity even increased by 6.47%. These samples showed the significantly higher ABTS activity both before and after frying. Different ABTS results from the DPPH (Table 4) could be explained with other reaction mechanism.

**Table 4.** The total phenol content (TPC) and antiradical activity DPPH and ABTS

| Sample | TPC, mg 100 $g^{-1}$DW | DPPH, mg 100 $g^{-1}$DW | ABTS, mg 100 $g^{-1}$DW |
|---|---|---|---|
| C_B | 147.19 ± 4.13a | 10.28 ± 0.34b | 6.13 ± 0.17e |
| SW_B | 147.56 ± 1.48a | 10.96 ± 0.34a | 8.27 ± 0.2c |
| IMW_B | 149.35 ± 4.84a | 11.01 ± 0.28a | 7.82 ± 0.4c |
| SE_B | 148.24 ± 1.95a | 10.88 ± 0.41a | 8.97 ± 0.25b |
| C_F | 102.78 ± 1.51c | 5.82 ± 0.14c | 6.27 ± 0.33e |
| SW_F | 103.7 ± 0.82c | 5.59 ± 0.17c | 7.16 ± 0.12d |
| IMW_F | 98.69 ± 1.06d | 5.24 ± 0.13d | 6.98 ± 0.2d |
| SE_F | 112.26 ± 2.48b | 5.73 ± 0.15c | 9.55 ± 0.17a |

*Values, marked with the same letter, are not significantly different at $P < 0.05$.

ABTS + radical is stable and is much more active than DPPH· radical. ABTS radical cation reactions with antioxidant is faster than the millisecond (Naik et al., 2003). ABTS reacts with most of the antioxidants, it does not affected by the ionic strength and is used to determine both hydrophilic and hydrophobic antioxidant activity (Martysiak-Żurowska & Wenta, 2012). Also the results of a variety of foods suggest that ABTS assay better reflects the antioxidant contents than DPPH assay and the correlation between antioxidant capacities detected by ABTS and DPPH assays was strong in fruits and beverages, but lower in vegetables. Most analysed vegetables showed much lower antioxidant capacities as measured by DPPH assay relative to ABTS assay (Floegel et al., 2011). Also experiments about potatoes showed that the ABTS value did not change significantly after frying of crisp (Kita et al., 2013).

**Vitamin C content**

The vitamin C content in all samples during frying decreased significantly ($P < 0.05$). Comparing the vitamin C content of potato samples after treatment with various lovage extracts the highest ascorbic acid content was found in the sample, which was sprayed with a water extract, while during frying in oil the highest ascorbic acid content remained in the sample, which was sprayed with lovage ethanol extract (Table 4). Regarding correlation between vitamin C content and acrylamide formation the authors Cheng et al. (2013) in their review paper gave the conclusions from the investigations of other researchers that there is a relatively weak reduction of the acrylamide formation by the addition of ascorbic acid to a potato model. Analysing the data of our experiment there can be found a tendency that higher vitamin C content in samples sprayed and immersed in water before frying could influence the formation of lower acrylamide content after frying although some more experiments are necessary to prove such hypothesis.

**The hardness of French fries**

Comparing the effects of the extract on the French fries straw hardness, it was determined that the least difference in hardness of a straw from a control sample was in the sample that was sprayed with water extract (Table 5). The highest maximal breaking force of fried potatoes was detected for sample IMW, but the lowest for sample SE.

**The acrylamide content**

The lowest acrylamide content was found in sample, which was sprayed with lovage-water extract. Also to a second sample, which was soaked in water, detected acrylamide content was significantly lower than in the control sample. But in sample sprayed with an alcohol solution acrylamide content after frying was even higher than to the control sample (Table 5).

This phenomenon could be explained with the reactions between ethanol and some chemical components of potatoes and lovage, and formation of chemical components which can join into the reactions of acrylamide formation.

**Table 5.** The vitamin C content, hardness of straws, and acrylamide content in blanched potatoes and French fries treated with different lovage extracts

| Sample | Ascorbic acid, mg 100 $g^{-1}$ DW | Hardness of straws, N | Acrylamide content, μg $kg^{-1}$ dryweight |
|---|---|---|---|
| C_B | 39.05 ± 2.63b,c | - | - |
| SW_B | 55.35 ± 1.48e | - | - |
| IMW_B | 50.01 ± 2.83d | - | - |
| SE_B | 46.55 ± 0.00d | - | - |
| C_F | 33.18 ± 1.34a | 17.79 ± 4.66a | 527.6 ± 12c |
| SW_F | 35.70 ± 0.66a,b,c | 18.03 ± 6.25a | 349.2 ± 9a |
| IMW_F | 34.93 ± 0.00a,b | 19.77 ± 4.29a | 380.9 ± 10b |
| SE_F | 40.00 ± 1.38c | 15.40 ± 5.26a | 584.9 ± 11d |

Similarly the authors Cheng et al. (2013) summarized that the preparative procedure, which often includes heat-assisted extraction or concentration steps, may change the composition of the extract, thus making it different from the raw material or the representative component, especially for heat unstable antioxidants. Also, since the extract is relatively complicated compared to pure compounds, the effect of co-extracts and interaction between representative components and the co-extracts should be taken into consideration. Previously, food researchers and technologists applied both antioxidative extracts and pure antioxidants to inhibit acrylamide generation. But both positive and negative results had been obtained (Cheng et al., 2013).

## CONCLUSIONS

Summarizing the results, it can be concluded that the best method for potato straw treatment is the spraying with lovage-water extract. Potato straws processed with this method contained by about 32% less acrylamide than the control sample, thus it can be concluded that treatment with lovage-water extract has a positive effect on the acrylamide content reduction in French fries and is a basis for further research on the detection of the optimal lovage-extract concentration.

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# Gas chromatography–mass spectrometry study of lipids in northern berries

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**Abstract.** Wild berries from forests and bogs of Northern Europe are an excellent source of natural antioxidants, vitamins and fatty acids, all of which are substances with high biological activity. This study investigates lipids extracted from fresh and powdered berries, using low-polarity solvents (chloroform, diethyl ether and others) and a mixture of chloroform and methanol. Berry lipids were analysed by gas chromatography–mass spectrometry. The following berries were analysed: blueberry (*Vaccinium myrtillus* L.), bilberry (*Vaccinium uliginosum* L.), two cultivars of highbush blueberry (*Vaccinium corymbosum* L.), lingonberry (*Vaccinium vitis-idaea* L.), cloudberry (*Rubus chamaemorus* L.), black crowberry (*Empetrum nigrum* L.), cranberry (*Vaccinium oxycoccos* L.) and rowanberry (*Sorbus aucuparia* L.). One hundred and elevencompounds were identified and quantified in the 9 species of analysed berries. The lipid fraction contained compound classes like fatty acids, sterols, triterpenoids, alkanes, phenolic and carboxylic acids and carotenoids. All fresh berries contained high amounts of C18 unsaturated fatty acids (for example, up to 102 μg $g^{-1}$ of blueberries) and phytosterols (86 μg of β-sitosterol $g^{-1}$ of blueberries), and high amounts of benzoic acid were found in lingonberries (164 μg $g^{-1}$). The analysed berry lipid profiles were compared using the principal component analysis and hierarchical cluster analysis. The two analyses showed that the lipid profiles of the studied berries reflect their taxonomy.

**Key words:** Northern berries, lipids, extraction, GC/MS, chemotaxonomy.

## INTRODUCTION

Nowadays, there is a strong scientific evidence that diets based on plant foods are the healthiest (Kähkönen et al., 2001; Silva et al., 2013). Regular consumption of vegetables, fruits and berries is beneficial for the prevention and reduction of developing chronic diseases (Hooper & Cassidy, 2006).

Berries have high concentrations of polyphenols, antioxidants, vitamins and minerals (Nile & Park, 2014). A large number of studies have shown the health benefits of berries, especially their antioxidant activity (Kähkönen et al., 2001), role in cardiovascular health (Rodriguez-Mateos et al., 2013) their protection against cancer (Määttä-Riihinen et al., 2005), anti-inflammatory, cholesterol-lowering (Szakiel et al., 2012a; Joseph et al., 2014) and antimicrobial effects (Silva et al., 2013), and so on.

Despite the fact that the lipid content in berries is low, there are several studies indicating the presence of different low-polarity substance groups in berries (Zlatanov 1999; Yang et al., 2003; Hoed et al., 2009). Plants (and also berries) contain lipids that regulate protein synthesis, metabolic and many other processes in cells (Corte et al.,

2015). Lipids in berries have a significant function to protect the berry from outside factors (cuticular waxes), such as pathogens and environmental stresses (Corte et al., 2015). Lipids in berries can be found in the cytoplasm, or they can be bound to cellular membranes; moreover, significant amounts of lipids can be found in berry seeds (Järvinen et al., 2010). Many lipid groups in berries (unsaturated fatty acids, sterols, terpenoids and others) have high biological activity, and they differ from lipids in higher organisms (mammals); therefore, their consumption is important for human metabolism. For example, it has been shown that berry sterols (phytosterols) have the ability to reduce cholesterol levels in humans (Dulf et al., 2012).

Studies of lipids in berries began with analysis of cranberry composition and major lipid groups in these berries. Croteau and Fagerson (1969) investigated cranberry seed lipids and found neutral lipids, phospholipids, glycolipids and many free lipids, such as paraffins, aldehydes, alcohols, fatty acids, sterols and carboxylic acids (Croteau & Fagerson, 1971). More recent studies have concentrated on cutin composition and lipid content analysis in berry seed oils (Johansson et al., 1997; Zlatanov, 1999; Johansson et al., 2000; Oomah et al., 2000; Kallio et al., 2006; Hoed et al., 2009; Dulf et al., 2012). It has been found that seed oils contain high amounts of polyunsaturated fatty acids and phytosterols. However, the contents of other low-polarity substances is not so widely studied, and only a few studies have concentrated on the analysis of sterol composition in berries (Dulf et al., 2012; Szakiel et al., 2012a; Szakiel et al., 2012b) or tocopherols (Zadernowski et al., 2003; Matthaus & Ozcan, 2014). Non-traditional vegetable oils, such as berry oils, have become increasingly popular in health care because of their highly specific composition, high concentration of unsaturated fatty acids and antioxidants. Since polyunsaturated fatty acids cannot be synthesised in human body, they must be obtained from food (Hoed et al., 2009). Therefore, the concept of 'berry lipids' could have a high marketing potential. Another group of substances found in berry seed oils are triterpenoids and sterols (Yang et al., 2003). Studies have shown the anti-inflammatory, antiviral, wound-healing and anticarcinogenic properties of sterols and triterpenoids present in berry seed oils (Szakiel et al., 2012b).

Regular consumption of berries has an impact on inflammation markers and antioxidative capacity (Rodriguez-Mateos et al., 2013; Joseph et al., 2014). Berries contribute to healthy gut microbiome, may improve lipid profile of human plasma and reduce the risk of cardiovascular diseases (Yang & Kortesniemi, 2015).

The studies done on berry lipids until now have concentrated mostly on cranberries and blueberries, as these are the most common commercially cultivated berries worldwide. However, many wild berries common in Northern Europe and the Baltic countries are available for everyday consumption. Moreover, many of these berries have a significant potential for cultivation and thus also for isolation of biologically active extracts and substances. Considering the high biological activity of berry lipids, it is important to continue studies of their composition for the purposes of uncovering their potential uses, developing new processing approaches and supporting innovation in the applicability of berries and their extracts in food, as food additives, in cosmetics, etc.

The aim of this study was to analyse the lipid composition of the selected 9 species of wild and cultivated berries commonly growing in Latvia.

## MATERIALS AND METHODS

### Berry samples and their processing

Nine species of berries grown in Latvia were investigated: blueberry (*Vaccinium myrtillus* L.), bilberry (*Vaccinium uliginosum* L.), highbush blueberry (*Vaccinium corymbosum* L.), lingonberry (*Vaccinium vitis-idaea* L.), cloudberry (*Rubus chamaemorus* L.), black crowberry (*Empetrum nigrum* L.), cranberry (*Vaccinium oxycoccos* L.) and rowanberry (*Sorbus aucuparia* L.). Two cultivars of highbush blueberry (cv. Blue Ray and cv. Chippewa) were studied, while the rest are wild berries.

Blueberries, lingonberries and rowanberries were harvested in the summer/autumn period (July-October) of 2014 in Vidzeme (Latvia). The cultivated highbush blueberries (HB) were harvested in a local garden in the town of Saldus in mid-August 2014. Bilberries were harvested in Pieņu bog in July 2014. Cloudberries were harvested in the bogs belonging to Teiču Nature Reserve in September 2014. Black crowberries and cranberries were harvested in Kurzeme, the western part of Latvia, in forests surrounding the town of Liepāja in September 2014. After the berries have been harvested, they were washed with demineralised water to remove any possible contaminations (dirt, bugs, etc.), air-dried and transported to the storage where they were frozen to -20 °C. Berries were kept at -18°C and analysed within 5–7months.

Blueberries and lingonberries were dried by the company SilvExpo SIA, using Мусson-2 microwave lyophiliser drying equipment (by BÜCHI Labortechnik AG, Switzerland). Berries were dried in vacuum at a temperature not exceeding 50 °C until they contained a maximum of 8% total moisture. After the berries have reached a satisfactory moisture level, they were milled and sifted.

### Extraction of berry lipids

For the extraction of lipids from fresh berries, 50 g of the selected berries was crushed in mortar. The crushed berries were then mixed with 150 mL of Bligh-Dyer reagent ($CH_3OH$ (Labscan)/$CHCl_3$ (Chempur), 2:1). After 2 minutes of stirring, 50 mL of $CHCl_3$ was added. The mixture was poured in a glass bottle with a cap and sonicated for 40 minutes; the water in the ultrasound bath (Cole – Parmer, USA) was changed every 20 minutes to avoid evaporation of the solvents and heating up. After the sonication, the samples were equilibrated to room temperature. 50 mL of $H_2O$ was added, and the samples were filtered. The final ratio of the solvents in the mixture was $CH_3OH$:$CHCl_3$:$H_2O$, 2:2:1. The berry residues in the filter were extracted again with 100 mL $CHCl_3$ and sonicated for another 40 minutes as before. The process was repeated twice. All the extracts were pooled in a separation funnel to separate the $CH_3OH$:$H_2O$ and $CHCl_3$ phases. The $CHCl_3$ phase was gathered and dried with $Na_2SO_4$ (Enola). The extract was filtered once again and rotary-evaporated at 40 °C max. until it formed thick syrup (Bligh & Dyer, 1959).

For the extraction of lipids from dried berries a similar approach to above was used, applying solvents with different polarities (hexane (polarity index 0.1), petroleum ether (polarity index 0.1), diethyl ether (polarity index 2.8), ethyl acetate (polarity index 4.4), and chloroform (polarity index 4.1)) (Labscan, Czech Republic). The weighed-out berry powder was mixed with 50 mL of the chosen solvent and sonicated for 20 minutes. The extract was left to shake for 24 hours, then sonicated for 20 minutes again and filtered. The filter paper together with the berry residues was extracted again with 50 mL solvent

by sonicating the mixture for 20 minutes, then filtering. The process was repeated twice. All extracts were pooled and evaporated until dry. The dry residue was then dissolved in 10 mL $CHCl_3$ and stored at -20 °C.

**Sample preparation and gas chromatography–mass spectrometry analysis**

The obtained berry extracts were evaporated to the amount of dry residue constituting ~20 mg in each sample. After the evaporation, the sample was kept in an exicator for 1 hour to make sure that it does not contain any residues of water. The dry sample was dissolved in 1 mL of acetonitrile (Sigma-Aldrich, USA), and 0.2 mL of N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) (Sigma-Aldrich, USA) was added. The sample was then heated at 60 °C for 1 hour. 0.5 mL internal standard was added to the sample. Palmitic acid methyl ester and dinonyl phthalate were used as an internal standard at the concentration of 150 mg $L^{-1}$ (Sigma-Aldrich). The sample was mixed in a 1.5 mL chromatography vial.

The parameters and chromatography settings can be seen in Table 1. The identification of the compounds separated in the GC was done using Perkin Elmer TurboMass v 6.0.0.1811 software, which uses NIST MS Search 2.0 spectral library. Several spectra of substances that could not be identified using the built-in library were identified comparing with the previously published spectra by other authors: sterols were identified according to Brooks et al., 1968, and Yang et al., 2003, whereas fatty acids were identified according to Mjøs 2004.

**Table 1.** Equipment and parameters used in the gas chromatography–mass spectrometry analysis

| Gas chromatograph–mass spectrometer Clarus 680/Clarus SQ8 (Perkin Elmer, USA) | |
|---|---|
| Column | Perkin Elmer Elite-5MS (5% diphenyl + 95% dimethyl polyoxane) 30 m x 0.25 mm x 0.25 μm. Working temperature range: +60 ° to +350 °C |
| Thermostat | Temperature programme: +75° (2 min) ramp to +130 °C with 20 °C $min^{-1}$, then to +300 °C with 4°C $min^{-1}$ held for 15 min |
| Carrier | Helium; flow rate 2.0 mL $min^{-1}$; split ratio 1:4 |
| Injector | Temperature +300 °C; injection done with auto-sampler; injected volume: 0.5 μL |
| Detector | Mass selective detector with quadrupole mass analyser; electron impact ionisation; energy: 70eV; ion source temperature: +300 °C; interface temperature: – +300 °C |
| Analysis time | 62.25 min |

**Statistics**

A triplicate determination for the Bligh-Dyer extraction was done to determine the standard deviation (SD) for the dry residue and selected compounds found in the fresh blueberries. Also, triplicate determination was done on the powdered blueberries and lingonberries. PCA analysis was done in order to group berry species based on compositional variability, and results visualized in component plot. PCA analysis was done to visualise the sample relations to each other. Hierarchical agglomerative cluster analysis was done to see how the chemical compounds link between the different analysed berries. Combining of clusters performed by Ward's method, were for each

cluster, the means for all variables and squared Euclidean distance are calculated. Accordingly to calculated summed distances clusters are merged where smallest increase of distances occurs. PCA and cluster analyses were done using Statistical Package for the Social Sciences (SPSS) software (IBM®, version 22).

## RESULTS AND DISCUSSION

The objective of the study was analysis of freely available lipids in 9 species of northern berries belonging to the *Ericaceae*, *Empetraceae* and *Rosaceae* families. Containing high concentrations of biologically active substances, these berries are traditionally used in ethnomedicine in the Baltic countries, and they have a perspective for use as nutraceuticals and for isolation of biologically active extracts or individual substances (Yang et al., 2011).

**Table 2.** Summary of sample extractions and corresponding dry residues

| Extraction type | Berries extracted, weight | Dry residue, mg $g^{-1}$ berries |
|---|---|---|
| **Bligh-Dyer (Bligh and Dyer, 1959) ($CH_3OH/CHCl_3$ 2:1)** | Fresh blueberry 50 g | 8.62 |
| | Fresh bilberry 50 g | 3.62 |
| | Fresh lingonberry 50 g | 5.68 |
| | Fresh cloudberry 50 g | 2.70 |
| | Fresh black crowberry 50 g | 4.64 |
| | Fresh cranberry 50 g | 3.72 |
| | Fresh rowanberry 50 g | 4.84 |
| | Fresh highbush blueberry (HB) | 2.36 |
| | (cv. Blue Ray(LKM), cv. Chippewa(MKM)) 50 g each | 3.06 |
| **Bligh-Dyer, 3 extractions** | Fresh blueberry 50 g each extr. | 8.43±0.32 |
| **Hexane** | Powdered blueberry 5 g | 46.22 |
| **Chloroform** | Powdered blueberry 5 g | 75.81 |
| **Petroleum ether** | Powdered blueberry 5 g | 40.15 |
| **Ethyl acetate** | Powdered blueberry 5 g | 52.53 |
| **Diethyl ether** | Powdered blueberry 5 g | 44.62 |
| **Diethyl ether, 3 extractions** | Powdered blueberry 5 g each extr. | 43.54±1.54 |
| **Diethyl ether, 3 extractions** | Powdered lingonberry 5 g each extr. | 32.03±0.52 |

To extract lipids from fresh berries, the main task was to dehydrate fresh, homogenised berries. To do this, an approach suggested by Bligh-Dyer (Bligh and Dyer, 1959) was used. A mixture of $CHCl_3$ and $CH_3OH$ was used, ensuring simultaneous dehydration of the berry mass and transfer of lipids to the $CHCl_3$ phase. The yield of lipid fraction using Bligh-Dyer extraction was from 2.70 mg dry residue $g^{-1}$ of cloudberries to 8.43 mg dry residue $g^{-1}$ of blueberries. The relative standard deviation for Bligh-Dyer extraction was determined to be ± 3.8%.

Considering the possible biological activity of extracts and results of other studies, analysis of freely available lipids were done (Fig. 1, Tables 3 and 4).

**Table 3.** Peaks of the blueberry extract chromatogram (Fig. 1), concentration of the substances found and their typical mass fragmentation

| Peak No. | Rt, min | Compound | C, μg $g^{-1}$ | Typical mass fragments |
|---|---|---|---|---|
| **1** | 4.70 | Lactic acid | 0.88 | 117(100), 73(90), 147(79), 191(16) |
| **2** | 4.87 | Hexanoic acid | 0.53 | 75(100), 73(82), 173(58), 117(24) |
| **3** | 5.83 | Heptanoic acid | 0.71 | 73(100), 75(92), 187(56), 117(48) |
| **4** | 6.82 | Benzoic acid | 6.13 | 179(100), 105(69), 77(54), 135(44) |
| **5** | 6.87 | Octanoic acid | 1.63 | 73(100), 75(67), 201(53), 117(51) |
| **6** | 8.39 | Nonanoic acid | 0.61 | 73(100), 74(31), 117(86), 215(62) |
| **7** | 10.15 | Decanoic acid | 1.12 | 73(100), 75(73), 117(62), 229(53) |
| **8** | 10.73 | Malic acid | 4.61 | 73(100), 147(35), 233(16), 75(12) |
| **9** | 12.16 | Undecanoic acid | 0.25 | 73(100), 75(70), 117(60), 243(50) |
| **10** | 12.38 | m-Hydroxybenzoic acid | 0.30 | 267(100), 73(58), 193(43), 223(40) |
| **11** | 14.36 | Dodecanoic acid | 0.64 | 117(100), 73(92), 257(73), 75(72) |
| **12** | 18.18 | D-fructose | 18.24 | 73(99), 204(44), 217(30), 147(24) |
| **13** | 18.95 | Tetradecanoic acid | 0.58 | 73(100), 117(68), 75(50), 285(43) |
| **14** | 20.90 | Phenyloctanoic acid | 3.16 | 75(100), 91(99), 117(95), 73(93) |
| **15** | 21.23 | Pentadecanoic acid | 0.44 | 73(100), 117(98), 75(68), 299(58) |
| **16** | 23.52 | Palmitic acid | 38.46 | 117(100), 73(94), 75(67), 132(49) |
| **17** | 25.66 | Heptadecanoic acid | 0.57 | 73(100), 117(68), 75(50), 327(43) |
| **18** | 27.04 | 9,12-Octadecadienoic acid | 26.44 | 73(100), 75(78), 67(61), 81(47) |
| **19** | 27.20 | 9,12,15-Octadecatrienoic acid | 102.10 | 73(100), 75(83), 79(75), 67(46) |
| **21** | 27.34 | Trans-11-octadecenoic acid | 51.16 | 73(100), 75(87), 117(71), 129(56) |
| **22** | 27.75 | Octadecanoic acid | 21.31 | 73(100), 117(83), 75(66), 129(43) |
| **23** | 31.74 | Eicosanoic acid | 5.52 | 73(100), 117(93), 75(62), 369(57) |
| **24** | 32.24 | Butyl 9,12-octadecadienoate | 7.96 | 73(100), 117(93), 75(62), 369(57) |
| **25** | 32.35 | Butyl 9,12,15-octadecatrienoic acid | 19.34 | 73(100), 75(86), 117(78), 129(51) |
| **26** | 34.52 | α-Monopalmitin | 0.33 | 28(100), 73(48), 371(46), 147(30) |
| **27** | 35.43 | Docosanoic acid | 0.72 | 117(100), 73(97), 75(60), 132(52) |
| **28** | 37.35 | Tetracosan-1-ol | 0.59 | 411(100), 75(97), 28(41), 43(41) |
| **29** | 38.45 | Squalene | 0.82 | 57(100), 43(93), 82(73), 55(67) |
| **30** | 38.89 | Tetracosanoic acid | 2.11 | 73(100), 117(92), 75(72), 43(50) |
| **31** | 40.65 | 1-Hexacosanol | 4.20 | 439(99), 440(36), 75(29), 57(16) |
| **32** | 42.22 | Octacosanal | 2.76 | 57(100), 43(82), 82(68), 55(56) |
| **33** | 43.54 | α-Tocopherol (Vitamin E) | 0.65 | 73(100), 502(94), 237(87), 236(65) |
| **34** | 43.76 | 1-Octacosanol | 0.40 | 468(100), 75(72), 57(61), 43(51) |
| **35** | 45.16 | Campesterol | 12.74 | 73(100), 129(84), 43(43), 75(38) |
| **36** | 45.33 | 1-Triacontanal | 0.95 | 57(100), 43(86), 82(82), 55(64) |
| **37** | 46.46 | β-Sitosterol | 84.64 | 129(100), 73(61), 43(49), 357(43) |
| **38** | 46.70 | β-Amyrin | 11.44 | 218(100), 203(40), 73(34), 75(25) |
| **39** | 47.31 | α-Amyrin | 5.30 | 218(100), 73(36), 189(31), 203(28) |
| **40** | 49.81 | Betulin | 0.53 | 73(100), 203(67), 189(43), 75(41) |
| **41** | 49.98 | Oleanolic acid | 14.85 | 203(100), 73(99), 202(78), 189(46) |
| **42** | 51.10 | Ursolic acid | 20.96 | 203(40), 73(99), 202(56), 133(55) |

**Table 4.** Lipid analysis of 9 chosen species of berries (ND – substance not detected; all values are expressed as μg of substance $g^{-1}$ of berries

| Rt, min | Compound | Cran-berry | Crow-berry | Cloud-berry | Lingon-berry | HB cv. Blue Ray | HB cv. Chippewa | Bil-berry | Blue-berry | Rowan-berry |
|---|---|---|---|---|---|---|---|---|---|---|
| 4.70 | Lactic acid | 1.16 | 2.91 | 0.91 | 2.45 | 2.52 | 1.30 | 2.23 | 0.88 | 0.43 |
| 4.87 | Hexanoic acid | 0.95 | 0.44 | 1.10 | 1.51 | 1.25 | 0.54 | 0.83 | 0.53 | 0.40 |
| 5.58 | m-Cresol | ND | ND | 1.26 | ND | ND | ND | ND | ND | ND |
| 5.72 | Benzyl alcohol | 0.69 | 0.72 | 22.21 | 3.00 | ND | ND | ND | ND | ND |
| 5.83 | Heptanoic acid | 0.47 | 0.73 | 0.39 | 0.58 | 0.85 | 0.41 | 0.57 | 0.71 | 0.74 |
| 6.35 | Pantoyl lactone | ND | ND | 0.53 | ND | ND | ND | ND | ND | ND |
| 6.82 | Benzoic acid | 37.08 | 2.84 | 68.41 | 164.40 | 4.40 | 0.64 | 0.51 | 6.13 | 2.90 |
| 6.87 | Octanoic acid | 0.87 | 0.32 | 0.45 | 1.31 | 1.86 | 1.00 | 0.67 | 1.63 | 1.05 |
| 7.01 | Phosphoric acid | 3.09 | ND | ND | 3.31 | ND | ND | 0.65 | 13.67 | 2.13 |
| 7.04 | Glycerol | ND | 2.65 | 4.77 | ND | 0.18 | 0.14 | 0.08 | 0.28 | ND |
| 7.48 | Phenylacetic acid | ND | 0.49 | ND | ND | 0.13 | 0.25 | 0.27 | 0.23 | ND |
| 7.64 | Succinic acid | 1.91 | 0.82 | 4.56 | 0.54 | 0.11 | 0.17 | 0.22 | 0.32 | 2.89 |
| 7.73 | Pyrocatechol | 0.08 | 4.64 | 0.13 | ND | ND | 0.08 | 0.13 | 0.25 | ND |
| 7.81 | Methylsuccinic acid | 0.61 | 0.67 | 0.63 | 0.28 | 0.33 | 0.15 | 0.27 | 0.25 | ND |
| 8.03 | Benzenepropanol | ND | ND | 0.54 | ND | ND | ND | ND | ND | ND |
| 8.20 | Fumaric acid | 0.32 | 0.26 | 0.36 | ND | ND | ND | 0.12 | 0.08 | ND |
| 8.25 | o-Toluic acid | ND | ND | ND | ND | 0.59 | 0.13 | ND | ND | 0.25 |
| 8.39 | Nonanoic acid | 0.70 | 0.67 | 0.34 | 1.43 | 1.40 | 0.44 | 0.65 | 0.61 | 0.74 |
| 8.47 | m-Toluic acid | ND | ND | ND | ND | 0.40 | 0.08 | ND | 0.08 | ND |
| 9.13 | Glutaric acid | 0.39 | 0.21 | 0.48 | ND | ND | 0.09 | 0.22 | 0.22 | ND |
| 9.43 | Hydrocinnamic acid | ND | 0.18 | 0.21 | 0.24 | 0.13 | 0.08 | 0.13 | 0.19 | 0.21 |
| 9.60 | 2-Deoxytetronic acid | ND | 0.42 | ND | 0.41 | 0.24 | 0.23 | 0.46 | 0.34 | ND |
| 9.65 | Cinnamic acid | 0.22 | ND | 8.54 | 1.42 | ND | ND | ND | ND | ND |
| 9.98 | 9-Decenoic acid | 0.16 | 0.22 | ND | 0.37 | 0.18 | 0.10 | 0.17 | 0.20 | ND |
| 10.15 | Decanoic acid | ND | 0.46 | 0.51 | 0.79 | 2.22 | 1.51 | 0.48 | 1.12 | 1.00 |

*Table 1 (continued)*

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 10.73 | Butanedioic acid | 41.68 | ND | 10.57 | 0.44 | ND | ND | 3.37 | 4.61 | 0.25 |
| 11.14 | Salicylic acid | 0.21 | ND | 0.17 | 1.61 | ND | ND | 0.14 | 0.16 | ND |
| 11.47 | Terpinol | 0.15 | ND | ND | ND | ND | ND | ND | ND | ND |
| 11.47 | p-Anisic acid | ND | ND | 0.18 | 0.33 | ND | ND | ND | ND | ND |
| 11.74 | Vanillin | ND | 0.26 | 0.11 | 0.08 | ND | 0.08 | ND | 0.16 | ND |
| 11.99 | 10-Undecenoic acid | ND | 0.29 | ND | ND | 0.23 | 0.10 | 0.17 | 0.27 | 0.19 |
| 12.02 | Trans-Cinnamic acid | 0.48 | ND | 6.95 | 5.95 | ND | ND | ND | ND | ND |
| 12.16 | Undecanoic acid | 0.20 | 0.27 | 0.13 | 0.35 | 1.15 | 0.13 | 0.26 | 0.25 | 1.06 |
| 12.38 | m-Hydroxybenzoic acid | 0.26 | 0.29 | 0.18 | 0.52 | 0.28 | 0.16 | 0.29 | 0.30 | 0.28 |
| 12.73 | β-Phenyllactic acid | 0.14 | 0.21 | 0.23 | ND | ND | ND | 0.13 | ND | ND |
| 13.23 | Pimelic acid | 0.35 | ND | 0.17 | 0.25 | ND | ND | ND | ND | ND |
| 13.75 | p-Salicylic acid | 0.26 | 1.02 | 0.91 | 1.06 | ND | 0.15 | 0.14 | 0.17 | 0.36 |
| 13.97 | Vanillic alcohol | ND | 0.48 | ND | ND | ND | ND | ND | ND | ND |
| 14.00 | 4-Hydroxyphenylacetic acid | 0.15 | ND | 0.21 | ND | ND | ND | ND | ND | ND |
| 14.36 | Dodecanoic acid | 0.87 | 0.83 | 1.47 | 1.92 | 1.72 | 0.36 | 1.06 | 0.64 | 5.19 |
| 15.40 | Octanedioic acid | 0.46 | ND | 0.53 | 0.37 | ND | ND | ND | ND | ND |
| 16.50 | 9-tridecenoic acid | 0.36 | ND | ND | 0.63 | ND | ND | 0.41 | 0.21 | ND |
| 16.61 | n-Tridecanoic acid | 0.17 | ND | ND | ND | ND | ND | ND | 0.19 | 1.41 |
| 16.91 | Vanillic acid | 0.60 | 1.02 | 0.45 | 1.40 | ND | ND | ND | ND | ND |
| 17.71 | Nonadioic acid | 0.65 | 0.22 | 0.55 | 1.49 | ND | ND | 0.15 | 0.17 | ND |
| 18.18 | D-fructose | ND | 2.90 | ND | ND | ND | ND | ND | ND | ND |
| 18.21 | Protocatechuic acid | ND | 0.46 | 0.09 | ND | ND | ND | 0.08 | ND | ND |
| 18.23 | Citric acid | 1.01 | ND | ND | 0.25 | ND | ND | ND | ND | ND |
| 18.65 | 9-Tetradecenoic acid | ND | ND | 0.51 | ND | ND | ND | ND | ND | ND |
| 18.95 | Tetradecanoic acid | 0.73 | 0.70 | 1.64 | 1.65 | 5.76 | ND | 0.41 | 0.58 | 9.72 |
| 20.05 | Syringic acid | ND | 1.58 | ND | ND | 0.12 | 0.09 | ND | ND | ND |
| 20.49 | Ferulic acid | 0.16 | ND | 0.68 | ND | ND | ND | ND | ND | ND |
| 20.90 | Phenyloctanoic acid | 0.39 | 0.38 | 0.38 | 0.96 | 0.48 | 0.20 | 0.35 | 3.16 | ND |

*Table 1 (continued)*

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 21.01 | p-Coumaric acid | 0.33 | ND | 5.32 | 1.10 | ND | ND | ND | 0.08 | ND |
| 21.23 | Pentadecanoic acid | 0.40 | 0.27 | ND | 0.62 | 2.31 | 0.26 | 0.27 | 0.44 | 1.55 |
| 22.69 | 9-Hexadecenoic acid | ND | ND | 0.13 | ND | ND | ND | ND | ND | ND |
| 23.24 | 11-Hexadecenoic acid | ND | ND | 4.44 | ND | ND | ND | ND | ND | ND |
| 23.52 | Hexadecanoic acid | 16.39 | 17.07 | 12.17 | 23.48 | 45.80 | 14.62 | 21.41 | 38.46 | 39.49 |
| 24.45 | Isoferulic acid | 0.08 | ND | 1.45 | 0.43 | ND | 0.23 | ND | ND | ND |
| 25.47 | Caffeic acid | ND | ND | 0.25 | ND | ND | ND | ND | ND | ND |
| 25.66 | Heptadecanoic acid | 0.46 | 0.37 | 0.30 | 0.64 | 1.41 | 0.43 | 0.44 | 0.57 | 1.53 |
| 27.04 | 9,12-Octadecadienoic acid | 19.81 | 10.41 | 8.68 | 22.81 | 1.99 | 5.01 | 17.83 | 26.44 | 1.29 |
| 27.20 | Trans-9-Octadecenoic acid | 32.16 | 27.14 | 23.28 | 62.83 | 20.54 | 13.82 | 40.41 | 102.10 | 3.86 |
| 27.34 | Trans-11-Octadecenoic acid | 10.88 | 8.98 | 1.42 | 22.71 | 17.98 | 7.03 | 16.12 | 51.16 | 6.26 |
| 27.75 | Octadecanoic acid | 5.86 | 4.23 | 3.78 | 6.47 | 13.97 | 5.00 | 8.85 | 21.31 | 11.42 |
| 29.02 | Linoleic acid | 11.17 | 6.55 | 1.74 | 14.39 | 0.50 | 3.44 | 10.87 | 0.08 | ND |
| 29.79 | Nonadecanoic acid | ND | 0.19 | ND | ND | 0.60 | 0.35 | 0.24 | 0.18 | 1.11 |
| 31.19 | 11-Eicosenoic acid | 0.53 | 0.35 | 0.43 | 0.40 | 0.65 | 0.20 | 0.19 | ND | ND |
| 31.74 | Eicosanoic acid | 2.63 | 3.33 | 1.05 | 13.31 | 2.16 | 4.81 | 4.21 | 5.52 | 1.57 |
| 32.24 | Butyl 9,12-octadecadienoate | 1.76 | 0.29 | 0.34 | 4.78 | 1.44 | 1.65 | 3.46 | 7.96 | ND |
| 32.35 | Butyl 9,12,15-octadecatrienoic acid | 3.44 | 0.30 | 0.58 | 7.98 | 2.73 | 2.71 | 7.25 | 19.34 | ND |
| 32.57 | Butyl 11-octadecenoic acid | 2.24 | ND | 0.13 | 0.76 | 0.82 | 0.64 | 1.26 | 5.28 | ND |
| 32.90 | Pentacosane | ND | 0.20 | 0.68 | ND | ND | 0.20 | 0.54 | ND | ND |
| 33.61 | Heneicosanoic acid | 0.38 | 0.22 | 0.08 | 0.24 | 0.34 | 0.28 | 0.29 | ND | ND |
| 33.83 | 1-Docosanol | 0.14 | 2.71 | 0.87 | 1.13 | 0.18 | 0.15 | ND | ND | 0.77 |
| 34.52 | α-Monopalmitin | 0.37 | 0.56 | 0.39 | 0.72 | 0.27 | 0.24 | 0.30 | 0.33 | 0.46 |
| 35.34 | Tetracosanal | ND | 2.10 | ND | 0.46 | ND | ND | 0.48 | ND | ND |
| 35.43 | Docosanoic acid | 2.40 | 0.52 | 0.76 | 2.72 | 1.31 | 1.04 | 0.85 | 0.72 | 0.50 |
| 35.62 | 1-Tricosanol | ND | 0.37 | 0.11 | ND | ND | ND | ND | ND | 0.20 |
| 36.55 | Heptacosane | ND | 0.45 | 3.65 | 0.31 | ND | ND | 1.42 | 0.42 | 0.48 |
| 37.19 | Tricosanoic acid | 0.43 | 0.22 | 0.15 | 0.62 | 0.26 | 0.28 | 0.47 | ND | ND |
| 37.35 | Tetracosan-1-ol | 0.33 | 14.50 | 2.43 | 1.63 | 0.36 | 0.26 | 0.33 | 0.59 | 1.91 |

*Table 1 (continued)*

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 37.93 | α-Monostearin | 0.32 | 0.48 | 0.25 | 0.70 | 0.13 | 0.10 | 0.32 | 0.35 | 0.12 |
| 38.45 | Squalene | 1.17 | 0.87 | 0.37 | 2.04 | 1.16 | 0.54 | 0.82 | 0.82 | 1.15 |
| 38.89 | Tetracosanoic acid | 3.35 | 1.74 | 0.75 | 2.50 | 0.24 | 0.72 | 5.91 | 2.11 | ND |
| 39.03 | 1-Pentacosanol | ND | 0.54 | 0.22 | ND | 0.09 | 0.12 | 0.21 | 0.28 | 0.31 |
| 39.96 | Nonacosane | ND | 1.55 | 0.74 | 2.48 | 0.12 | 0.45 | 0.22 | ND | 4.48 |
| 40.52 | Pentacosanoic acid | 0.34 | 0.54 | ND | 0.24 | ND | 0.08 | ND | ND | ND |
| 40.65 | 1-Hexacosanol | ND | 3.86 | 2.98 | 0.82 | 0.45 | 0.58 | 0.97 | 4.20 | 0.86 |
| 41.34 | γ-Tocopherol | 0.13 | 0.20 | 0.15 | 0.23 | 0.61 | 0.45 | 0.13 | 0.25 | 0.25 |
| 42.11 | Hexacosanoic acid | 2.00 | 0.49 | 0.40 | 2.92 | ND | ND | 0.68 | ND | ND |
| 42.22 | Octacosanal | 0.66 | 0.90 | 0.21 | 1.35 | 0.39 | 0.82 | 13.96 | 2.76 | ND |
| 43.32 | Chlorogenic acid | ND | ND | ND | ND | 0.24 | 1.37 | ND | ND | ND |
| 43.54 | α-Tocopherol | 1.13 | 0.77 | 3.51 | 2.22 | 1.44 | 1.19 | 0.78 | 0.65 | 3.34 |
| 43.76 | 1-Octacosanol | ND | 1.17 | 1.10 | 0.21 | 0.56 | 0.72 | 1.72 | 0.40 | 0.86 |
| 45.16 | Campesterol | 2.98 | 1.05 | 0.52 | 3.91 | 0.51 | 0.36 | 0.47 | 12.74 | ND |
| 45.33 | 1-Triacontanal | 1.30 | 0.31 | ND | 1.89 | 0.32 | 0.50 | 0.56 | 0.95 | ND |
| 45.88 | α-Tocopherolhydroquinone | ND | ND | ND | ND | 0.14 | 0.11 | ND | ND | ND |
| 46.46 | β-Sitosterol | 6.48 | 6.25 | 4.23 | 11.87 | 8.59 | 7.25 | 8.52 | 84.64 | 6.21 |
| 46.70 | β-Amyrin | 0.38 | 2.11 | 0.87 | 1.31 | 1.03 | 0.86 | 1.14 | 11.44 | 2.14 |
| 47.31 | α-Amyrin | 0.55 | 11.05 | 0.14 | 1.79 | 1.31 | 0.88 | 0.16 | 5.30 | 2.82 |
| 47.42 | Cycloartenol | 0.23 | ND | 1.57 | 2.39 | ND | 3.65 | ND | ND | 0.98 |
| 48.02 | Lanosterol | ND | ND | ND | 4.92 | ND | ND | ND | ND | ND |
| 48.02 | Nonacosanoic acid | 0.81 | ND | 0.40 | ND | ND | ND | ND | ND | ND |
| 48.05 | Triacontanoic acid | 0.81 | ND | ND | ND | ND | ND | ND | ND | ND |
| 48.63 | Lupeol | 0.20 | 1.37 | ND | 0.77 | ND | ND | 0.33 | 0.51 | ND |
| 48.82 | Erythrodiol | ND | 1.81 | ND | 0.80 | ND | ND | ND | ND | ND |
| 49.51 | Uvaol | ND | 3.61 | ND | 0.91 | ND | ND | ND | ND | ND |
| 49.81 | Betulin | ND | ND | 0.09 | 0.88 | 0.42 | 0.36 | 0.57 | 0.53 | 1.33 |
| 49.98 | Oleanolic acid | 9.98 | 11.32 | 1.09 | 6.67 | 0.52 | 1.42 | 0.11 | 14.85 | 7.02 |
| 51.10 | Ursolic acid | 53.18 | 44.15 | 6.24 | 30.54 | 1.31 | 3.94 | 26.76 | 20.96 | 4.94 |

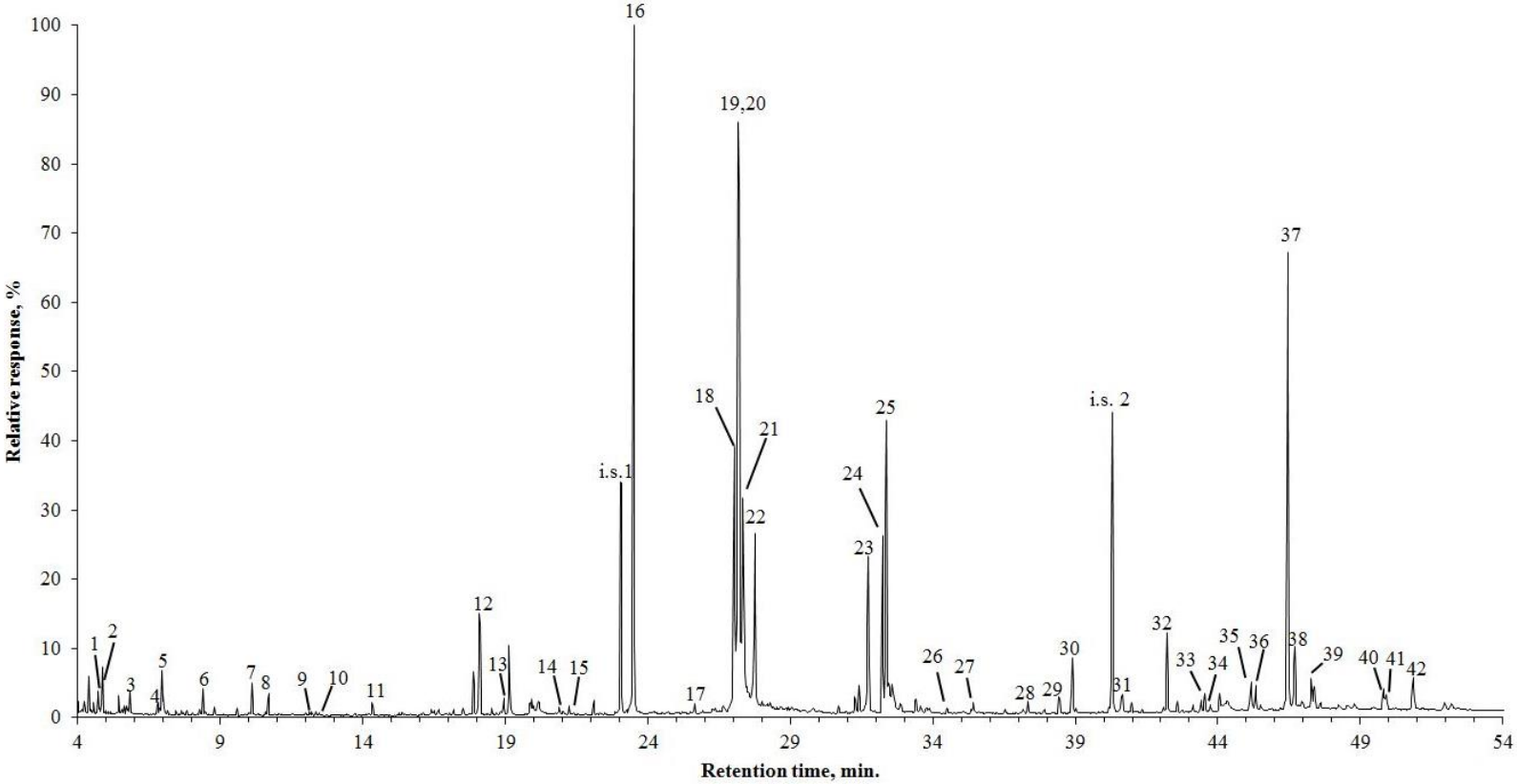


**Figure 1.** Chromatograms of blueberry Bligh-Dyer extract (peak numbers as indicated in Table 3).

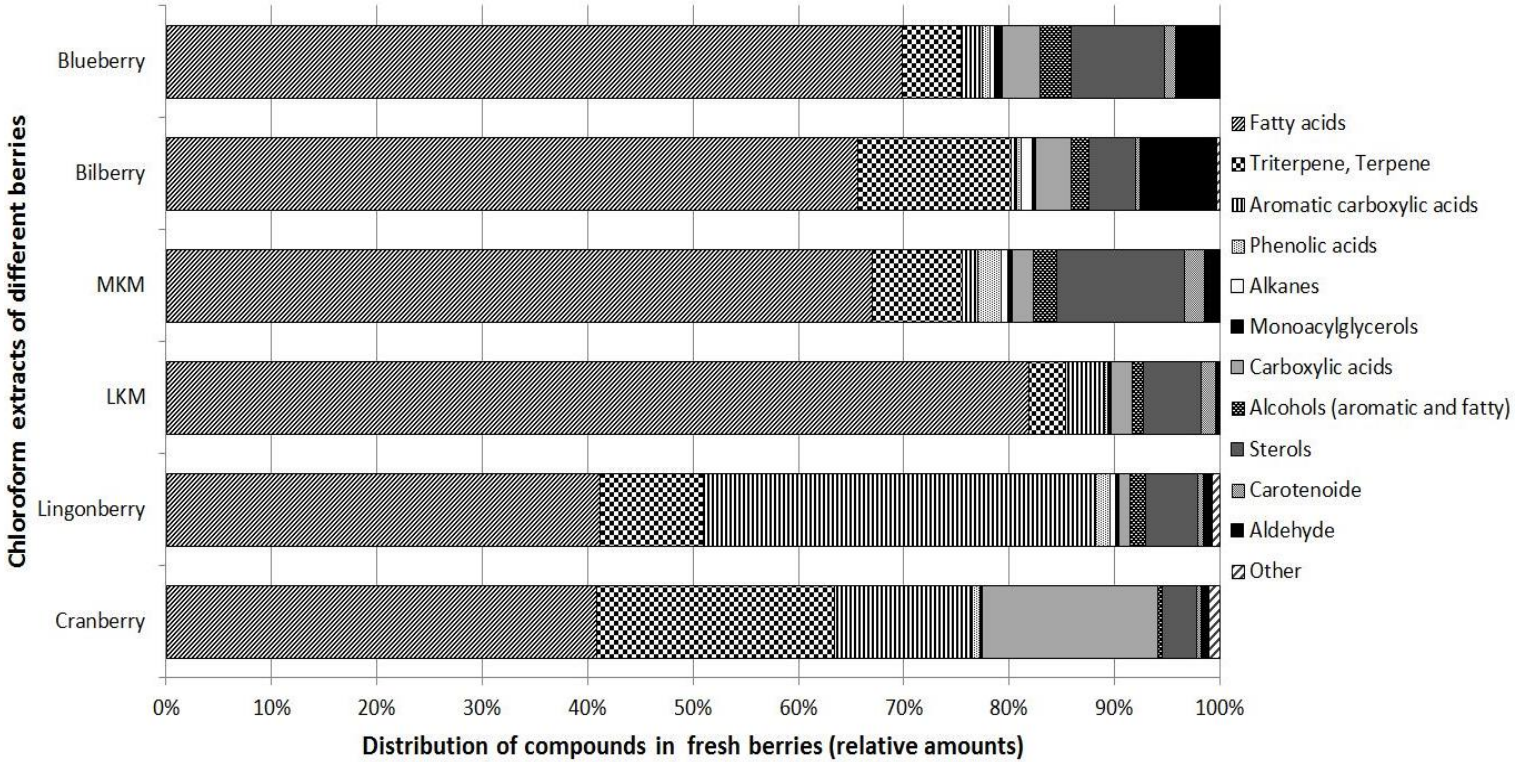


**Figure 2.** Distribution of compound classes in the studied berries.

In recent years, berry powders (dried, lyophilised berries) are used more frequently in the food industry (Nile & Park 2014). To estimate the most abundant lipids in berry powders, single-solvent extraction was tested using 5 different solvents, considering their possible extraction efficiency, perspectives of their application at an industrial scale and environmental aspects of their application. Single-solvent extraction from berry powder gives much higher yields of dry residue than the use of $CHCl_3$ and $CH_3OH$ mixture. The highest concentration of dry residue was found in the powdered blueberry extracts with diethyl ether, giving 75.81 mg dry residue $g^{-1}$ of berries, while the highest yield of Bligh-Dyer dry residue was 8.62 mg $g^{-1}$ of berries (Table 2). Repeatability for the single-solvent extraction was estimated by doing 3 separate extractions on 2 different berry powders: blueberry and lingonberry. The repeatability was ± 3.54% and ± 1.64% respectively, which is similar to the repeatability of Bligh-Dyer extraction.

The substances with highest concentrations in the blueberry extracts were C18 unsaturated fatty acids (26.44–102.10 mg 100 $g^{-1}$ berries) (Table 4), which were also found in the previous studies (Johansson et al., 1997; Croteau & Fagerson, 1969; Dulf et al., 2012). Two of the peaks of unsaturated fatty acids overlapped (peaks 19 and 20, Fig. 1), and they were quantified as a single peak. The rest of the peaks had good separation.

Freely available lipids obtained using the Bligh-Dyer extraction method from the 9 chosen types of berries were determined. In total, 111 different substances were identified (Table 4) by comparing their mass spectra and retention index with the reference mass spectra and reference retention index (Mjøs, 2004). The highest numbers of substances were found in the cloudberry (86), lingonberry (79) and crowberry (78) extracts.

The lowest numbers of substances were found in the rowanberry (50), highbush blueberry cv. BlueRay (63) and blueberry (65) extracts. 70 substances were identified in the highbush blueberry cv. Chippewa extract and 73 and 75 substances – in the bilberry and cranberry extracts respectively. Substances like benzoic acid (0.64–164.40 μg $g^{-1}$ berries), nonanoic acid (0.34–1.43 μg $g^{-1}$), m-hydroxybenzoic acid (0.16–0.52 μg $g^{-1}$), squalene (0.37–2.04 μg $g^{-1}$), α-tocopherol (0.65–3.51 μg $g^{-1}$) and β-sitosterol (4.23–84.64 μg $g^{-1}$) were found in all berries in various concentrations. Some of the substances were found in one berry type only – for example, lanosterol in lingonberries (4.92 μg $g^{-1}$), m-cresol in cloudberries (1.26 μg $g^{-1}$), uvaol in crowberries (3.61 μg $g^{-1}$) and chlorogenic acid in both cultivars of highbush blueberry (0.24–1.37 μg $g^{-1}$) (Table 4). The substance with the highest concentration was benzoic acid (164.40 μg $g^{-1}$) in lingonberries. Also, all of the C18 unsaturated fatty acids were in high concentrations (up to 102.10 μg $g^{-1}$ of blueberries).

Single-solvent extractions were done on dry berry powders to find the best solvent for lipid extractions. Five solvents were used: hexane, petroleum ether, diethyl ether, ethyl acetate and chloroform. 22 peaks were identified and quantified (Table 5). The largest amount of substances was extracted using diethyl ether (2.9 mg $g^{-1}$ berry powder) and hexane (1.5 mg $g^{-1}$), the least amounts were extracted with petroleum ether (0.36 mg $g^{-1}$). Hexane and diethyl ether extracts contained large amounts of β-sitosterol (341.47 and 334.31 mg $g^{-1}$ berry powder). Diethyl ether extracts contained large amounts of C18 unsaturated fatty acids (101.98–818.52 μg $g^{-1}$) and malic acid (402.19 μg $g^{-1}$).

**Table 5.** Single-solvent extractions on powdered blueberry sample (all values expressed as μg of substance $g^{-1}$ of berry powder

| Substance | Hexane | Petroleum ether | Diethyl ether | Ethyl acetate | Chloroform |
|---|---|---|---|---|---|
| **Benzoic acid** | 65.97 | 22.81 | 64.74 | 16.90 | 49.04 |
| **Nonanoic acid** | 2.75 | 1.92 | 2.77 | 2.65 | 2.65 |
| **Butanedioic acid** | 0.00 | 0.00 | 402.19 | 0.00 | 0.00 |
| **Dodecanoic acid** | 2.80 | 1.78 | 4.32 | 1.92 | 3.35 |
| **Citric acid** | 0.00 | 0.00 | 77.33 | 0.00 | 0.00 |
| **Glucofuranoside** | 13.85 | 2.10 | 10.51 | 3.18 | 9.12 |
| **Palmitic acid** | 82.23 | 16.13 | 121.34 | 1.83 | 118.75 |
| **9,12-Octadecadienoic acid** | 75.15 | 5.59 | 320.75 | 718.16 | 223.80 |
| **9, 12, 15-Octadecatrieonoic acid** | 298.11 | 10.94 | 818.52 | 105.18 | 37.24 |
| **trans-11-Octadecenoic acid** | 53.61 | 2.58 | 101.98 | 36.65 | 65.51 |
| **Octadecanoic acid** | 12.77 | 2.58 | 41.65 | 2.87 | 2.70 |
| **Butyl 9,12-octadecadienoate** | 87.43 | 27.08 | 97.48 | 309.95 | 90.98 |
| **Butyl 9,12,15-octadecatrienoate** | 261.20 | 65.93 | 296.93 | 38.41 | 323.27 |
| **Butyl octadecanoate** | 34.52 | 11.54 | 39.22 | 10.25 | 30.88 |
| **Heptacosane** | 13.70 | 8.76 | 8.31 | 1.73 | 6.83 |
| **Nonacosane** | 15.05 | 2.01 | 15.14 | 3.21 | 15.22 |
| **Octacosanal** | 62.53 | 18.23 | 77.55 | 2.42 | 77.70 |
| **Triacontanal** | 31.15 | 3.14 | 27.83 | 1.86 | 36.62 |
| **β-Sitosterol** | 341.31 | 132.43 | 334.47 | 52.08 | 321.37 |
| **β-Amyrin** | 59.78 | 14.51 | 57.93 | 13.54 | 57.90 |
| **α-Amyrin** | 16.36 | 7.13 | 15.04 | 26.91 | 18.22 |
| **Betulin** | 8.58 | 3.84 | 15.51 | 17.91 | 24.63 |
| **Total, μg $g^{-1}$ of berries** | 1,538.85 | 361.02 | 2951.52 | 1367.6 | 1515.80 |

To evaluate whether the extractions give constant results, triplicate extraction of the berry powders using diethyl ether were done. Blueberry and lingonberry extracts were prepared, with triplicate determination for each. Lingonberry powder extract of diethyl ether contained high amounts of benzoic acid ($289.39 \pm 20.30$ μg $g^{-1}$), C18:3 unsaturated fatty acid ($417.76 \pm 46.1$ μg $g^{-1}$) and ursolic acid ($447.21 \pm 36.2$ μg $g^{-1}$). Relative standard deviation (RSD) for the 19 identified and quantified compounds in lingonberry powder was estimated to be between 1.95% and 12.57%. Triplicate blueberry powder diethyl ether extracts contained high amounts of malic acid ($105.10 \pm 7.5$ μg $g^{-1}$), C18:2 unsaturated fatty acid ($565.40 \pm 14.9$ μg $g^{-1}$) and β-sitosterol ($189.21 \pm 21.7$ μg $g^{-1}$). RSD for the 22 compounds with highest response found in blueberry diethyl ether extracts ranged from 1.3% to 12.2%.

Table 4 includes many substances that have not been described as part of berry lipids in any of the previous studies. For example, black crowberry lipids have not been described previously. Although several species of the chosen berries have been widely studied (especially the *Vaccinium* species), such a thorough description of free berry lipids has not been reported before. Substances like lactic acid, malic acid, nonanoic acid, decanoic acid and many others found in blueberry extracts have not been described previously. However, a few substances that could not be recognised were found in

addition to the identified compounds. No spectra similar to those that have been found could be matched with any of the reference spectra.

All the identified compounds of berry lipids can be divided into respective classes of organic substances, and a total of 11 classes of compounds were found (Fig. 2). The largest class in each type of berries except cloudberry was fatty acids (up to 82% of the total lipids). Blueberry, bilberry, both cultivars of highbush blueberries (LKM and MKM) and rowanberry have very similar profiles of compound classes (Fig. 2). Lingonberry and cloudberry have similar profiles: the fatty acid (41% and 30% respectively), triterpene (10% and 5%) and aromatic carboxylic acid (39% and 35%) classes give the same pattern for both types of berries. Black crowberry and cranberry also give similar patterns in composition profiles – both types of berries contain more triterpenes (35% and 25% respectively) than any other berries. Rowanberries are covered with waxy coating to protect from environmental stresses; they also have the highest amount of alkanes (5%), which are part of the berry cuticular waxes. Cloudberry and black crowberry extracts contained high amounts of alcohols and terpenes (Fig. 2).

The high content of fatty acids in the studied berries is due to the fact that berries have a lot of seeds, where the energy is stored in the form of fatty acids. Blueberry, bilberry and both cultivars of highbush blueberries (LKM and MKM) are closely related, which can also be seen in their compound class profiles.

Many of the berry lipids have high biological activity that might influence the application potential of lipid extracts.

The results of chromatographic analysis (Table 4) demonstrate significant differences amongst the studied berries. However, an open question is how the chemical composition reflects the biological taxonomy. Another open question is whether the growth environment can be considered as a major factor affecting the plant (berry) chemical composition.

Chemotaxonomy shows similarities in the chemical compositions of organisms in respect to their biological classification (Bisby et al., 1980). Chemotaxonomical analysis of the studied berries was done using the Principal Component Analysis (PCA) for the presence or absence of a chemical in the respective berry. Each compound was given a rank: 1 if the specific compound is present (component 2), 0 if absent (component 1). 2 components can explain the total variance of data by 61%. The PCA analysis shows two larger groups of berries, one consisting of cranberry, cloudberry and lingonberry, the second consisting of bilberry, blueberry, rowanberry, crowberry and both cultivars of highbush blueberries (Fig. 3). As expected, the two cultivars of highbush blueberry (LKM and MKM) are very similar to each other when their lipid composition is compared, as it is the same species of berries. Although distinguished as separate species, blueberry and bilberry are very closely related, and their lipid profiles are also similar. Cranberry and lingonberry come from the same genus, whereas cloudberry is from another family of plants. Cranberry, lingonberry and cloudberry have more similarities between each other than they have with, for example, any type of blueberries (positioned on the opposite sides of the axis).

Rowanberry and crowberry have similar lipid profiles with the blueberry species, even though this cannot be explained with the taxonomy of these berries, as rowanberry (*Rosaceae*) and crowberry (*Empetraceae*) belong to different families than blueberries (*Ericaceae*).

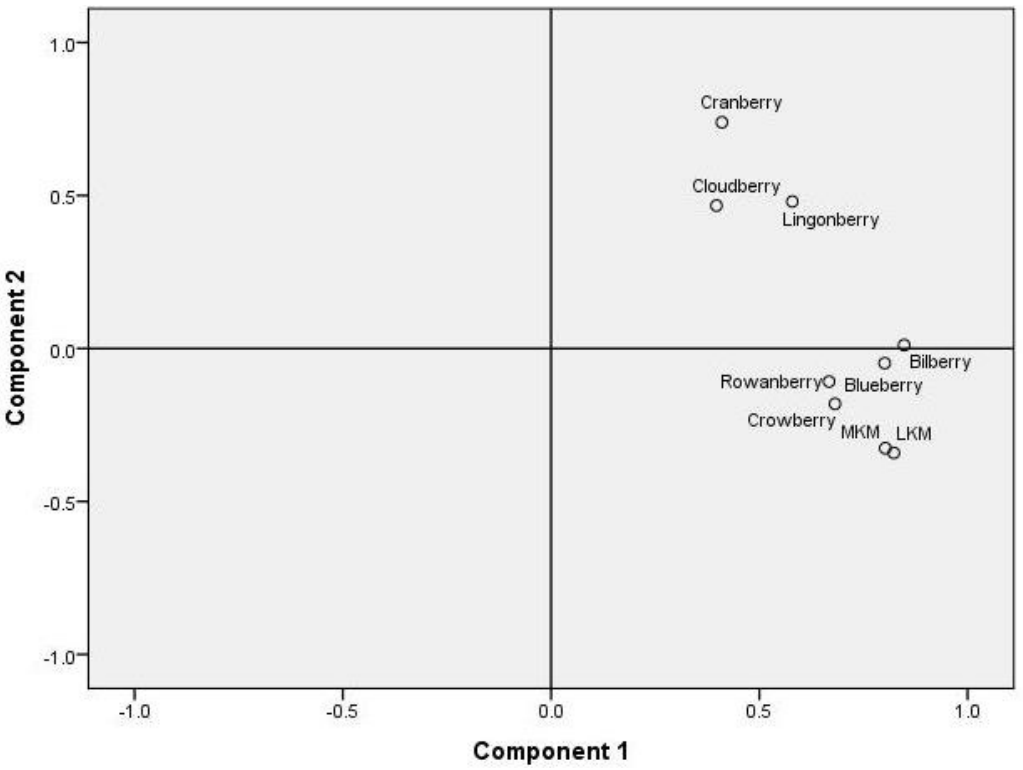


**Figure 3.** PCA analysis of the compounds present in the studied berries.

To support the results obtained by the PCA analysis even further, a hierarchical cluster analysis (HCA) was done to visualise the relations between different types of berries (Fig. 4). As confirmed by the PCA analysis, there are two distinct groups of berries, one consisting of cranberry, cloudberry and lingonberry, the second consisting of bilberry, blueberry, rowanberry, crowberry and both cultivars of highbush blueberries. In the dendrogram, it can be seen that rowanberries are remotely related to highbush blueberries, blueberries and bilberries, while not having many similarities with those berries. While PCA shows two base groups of berries, HCA demonstrates combination ways for each particular berry cluster, e.g. showing on this basis that in cranberry, lingonberry and cloudberry cluster cranberries are more similar to lingonberries within one cluster. Bilberries and blueberries, including both cultivars of highbush blueberry, show close relation between each other.

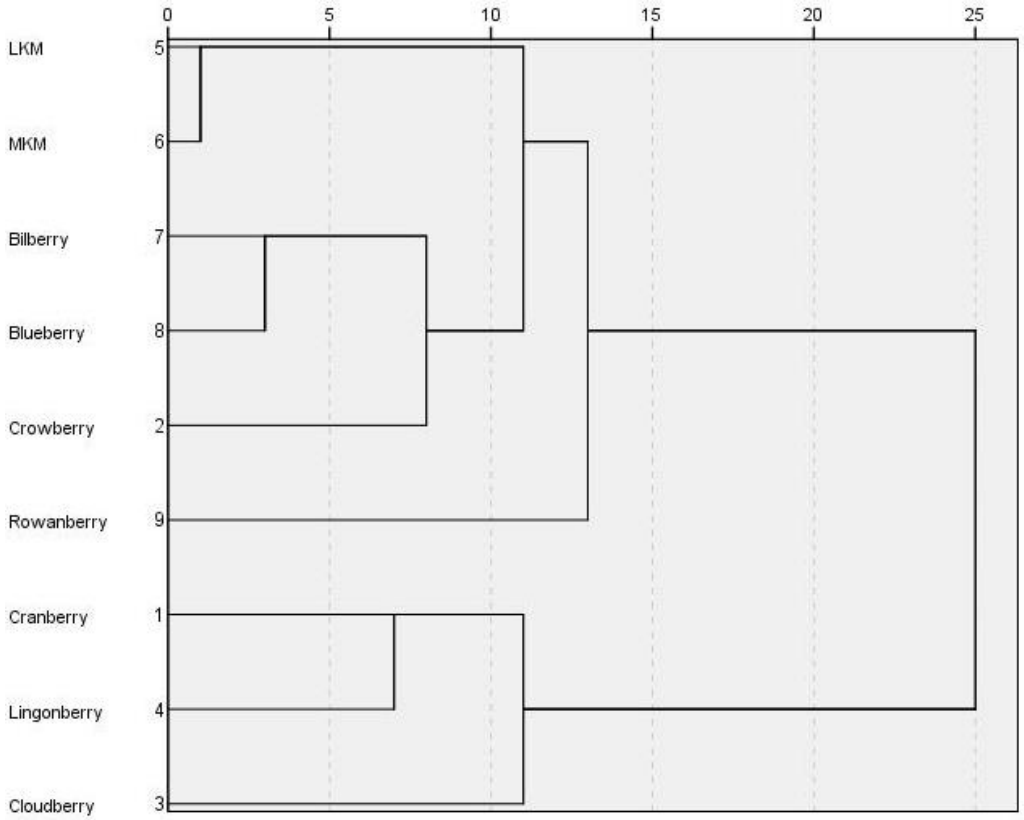


**Figure 4.** Hierarchical cluster analysis of the compounds present in the studied berries.

## CONCLUSIONS

Northern berries contain significant amounts of lipids, depending on the berry species, extraction conditions and solvents used in the extraction process. A lipid profile study was done for 9 species of berries common in Northern Europe and Latvia. 111 compounds were identified, many of which have not been described before as a part of berry lipids. The black crowberry lipid profile has not been studied before. The berry lipids found can be divided into 11 classes of organic compounds, for example, fatty acids, sterols, triterpenoids, carboxylic and phenolic acids and alkanes. The major group of lipids found in the studied berries was fatty acids. However, in respect to the functional significance and potential for application, sterols, triterpenoids and phenolic acids are of special interest. Lipids of some berries contain substances specific for the relevant species, for example, a high concentration of benzoic acid characterises the lipid pool of lingonberries.

The chemotaxonomic analysis of berry lipid profiles reflect their taxonomy using the principal component analysis (PCA) and hierarchical cluster analysis. as it demonstrated relations between the species (different cultivars) and genus. At the same time, no strict relation was seen between the families of berries, probably due to environmental factors (climatic conditions, growth substrate, harvest time, etc.). Berry

lipids are mainly extracted from berry seeds, and unsaturated fatty acids are used in many commercial products. This study has demonstrated the presence of a wide array of biologically active compounds (phytosterols and triterpenoids and others) that can support new fields of application of berry lipids, for example, as functional food, biopharmacy.

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# Egg yolk oil as a source of bioactive compounds for infant nutrition

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**Abstract.** Egg yolk oil is a natural source of bioactive compounds such as DHA, fat–soluble vitamins, lutein, phospholipids and cholesterol. These important compounds are also found in breast milk: DHA for infant brain development, lutein for eye health, vitamins A and E to support developing cells. Egg yolk oil naturally contains vitamin D which is required for a normal bone development. Fatty acid profile of egg lipids is also close to human milk. The aim of this study was to evaluate the conformity of egg yolk oil for infant nutrition. In this study egg yolk oil extracted from liquid egg yolk using two-stage solvent extraction with polar and non-polar solvents was used. Extracted egg yolk oil was analyzed for fatty acids, fat-soluble vitamins, lutein, phospholipids and cholesterol using GC and HPLC methods. Results were compared with the chemical composition of human breast milk and nutritional recommendations for infant feeding. Fatty acid profile of egg yolk oil was similar to breast milk in terms of palmitic, stearic, linoleic and α-inolenic acids. Egg yolk oil used in this study was high in DHA, but low in ARA. Vitamin A, D and E content was sufficient for infant biological needs. Lutein and phospholipid content in egg yolk oil was lower than their content in breast milk fats, but cholesterol in opposite was in much higher concentration than available in breast milk. Chemical composition of egg yolk oil still makes it an excellent source of bioactive compounds for infant nutrition.

**Key words:** egg yolk oil, infant nutrition.

## INTRODUCTION

There is no doubt that mother breast milk is the best diet for an infant. Breast milk contains all nutrients that are essential for normal development of the infant. But not all of mothers can or want to breast-feed their babies. For that reason artificial infant formulas containing many nutrients were created to fulfill biological needs of infants (Lawrence & Lawrence, 2016). All infant formulas try to replicate human milk in terms of nutritional and biological value. Some bioactive compounds, such as polyunsaturated fatty acids (PUFA), fat-soluble vitamins A, D and E, lutein, phospholipids and cholesterol are presented in lipids therefore the right choice of fats for infant formulas is so important (Berthold et al., 2005). In this study we evaluate the conformity of egg lipids (egg yolk oil) as a source of bioactive compounds for infant nutrition.

Bioactive compound content in human milk depends on the type of milk: colostrum, transitional and mature milk. Colostrum and transitional milk have a different and variable chemical composition and available for the infant for a very short period, but mature milk has more stable composition and is fed to infants for a longer time. Average fat content of mature milk is 4–5%. Breast milk fats provide 50% of total

calories consumed by infant (Sala-Vila et al., 2005). The content of fatty acids (FA) is very important in human breast milk, especially the content of polyunsaturated fatty acids (PUFA). PUFA content is about 1/5 from the total fatty acids (Lassek & Gaulin, 2014), where arachidonic (ARA) and docosahexaenoic (DHA) acids are the most important. PUFA content in breast milk depends mainly on mother's diet, wherein saturated fatty acid (SFA) content is relatively constant (Soleimani et al., 2013; Lassek & Gaulin, 2014). ARA and DHA play an important role for infant development, but their content in human milk is quite low and varies from 0.1 to 1.0% from total FA (Saphier et al., 2013). Both ARA and DHA are FA of phospholipids (PLs) and consumption of these FA by infant will depend on PL content in formulas (Heird, 2001). Egg yolk lipids are very close to human milk in terms of FA profile (Simopoulos & Salem, 1992) and contain about 10% of PL from total lipids (Ahn et al., 2006).

Without PUFA infants also need fat–soluble vitamins. Vitamins A, D and E are very crucial for infant development. Food must provide these essential vitamins to infants because human body cannot synthesize them.

Breast milk is rich in vitamin A, especially in the first postnatal days which probably relates to that fact that almost all children are born with very low stores of vitamin A. Later vitamin A content in human milk decreases (Fujita et al., 2011). Vitamin A is required for optimal health, growth and development of the infant therefore starting from 6 months of age consumption of vitamin A must be increased by additional supplement. Many children around the world are suffering from vitamin A deficiency that causes many deaths among babies (WHO, 2011). In the developed countries and countries where mothers consume a lot of leafy vegetables and other products rich in vitamin A (including eggs), deficiency of vitamin A in breast milk is not so actual, since vitamin A content in breast milk is correlated to its content in mothers' diet (Fujita et al., 2011). But vitamin A in high dosages can be toxic (Olson, 1989). There is a lot of studies about poisoning caused by vitamin A overdosing therefore vitamin A supplementation must be strongly controlled. Egg yolk naturally contains vitamin A and its content in eggs can be increased through hen's diet. Egg yolk oil can be used as a source of vitamin A for infant nutrition.

Vitamin E is extremely important in the early stages of life. Colostrum contains a huge amount of vitamin E while mature milk has much less. Being strong antioxidant the main function of vitamin E is to protect tissues from various destructive influences and stimulate infant immune system development. α-tocopherol is the most active form of vitamin E, but human milk has been found to contain β-, γ- and δ-tocopherols and γ-tocotrineols (Lima et al., 2014). Vitamin E in egg yolk lipids is presented by α-tocopherol and γ-tocopherol (Kovalcuks & Duma, 2013). There is a strong correlation observed between vitamin E content in eggs and in hen diet (Mori et al., 2003), so vitamin E is another example of adjustable bioactive compound.

Vitamin D helps to absorb calcium in human body and is required for a bone development of an infant. Lack of a vitamin D can cause rickets. Vitamin D also plays a role in muscle function and the immune system. Human breast milk contains insufficient amount of vitamin D therefore pediatricians recommend vitamin D as a peroral supplement (vitamin D droplets) for all breast-feed infants (Ballard & Morrow, 2013). But it needs to be aware with the dosage because vitamin D, similar to vitamin A, is toxic in high dosages. Instead of peroral supplement, food products rich in vitamin D, such as oily fish, eggs and others can be fed to the infant. But infants during the first year

of their life reluctantly consume such food and prefer liquid milk formulas. Vegetable fat-based artificial milk formulas do not contain vitamin D because vitamin D is an animal source compound. And usually vitamin D is added to infant formulas as a mono ingredient. Egg yolk oil, which is rich in natural highly bioavailable vitamin D, can be used in formulas and solve the problem with the absence of this vitamin.

Lutein is the major carotenoid found in breast milk. Lutein and its isomer are found in the neural retina and contribute to brain development. Similar to many other bioactive compounds, lutein content in human breast milk depends on mother's diet (Jewell et al., 2004). Eggs from hens fed by corn or green forage are rich in lutein. Lutein and other carotenoids are located in egg yolk lipids therefore egg yolk oil can be a good source of lutein for infant feeding. Usually infant formulas do not contain lutein since egg yolk lipids are not added (FSANZ, 2008).

The main phospholipids of human milk are phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Phospholipids provide not only ARA and DHA, but also choline – an essential compound for infant brain development (Schneider, 2010). It is well known that egg yolk lipids are rich in PLs, which contain 30% of PLs from total yolk lipids (Ahn et al., 2006). PC is the major phospholipid of egg yolk meaning that egg yolk lipids are one of the richest sources of choline.

A lot of health concerns relates to cholesterol. High content of cholesterol in food results in high cholesterol level in blood and may cause a heart coronary disease (Paik & Blair, 1996). But it is totally opposite in case of infants. Cholesterol contribute to development of brain and nervous system of the infant and therefore it is one of the major compounds for infant nutrition. Moreover, infants fed with breast milk containing high cholesterol have a normal metabolism of this compound in later life (Owen et al., 2008). Cholesterol content in breast milk depends on an individual and can be affected by many other parameters, such as mother's age, diet, season and place of residence (Kamelska et al., 2012). Infant formulas contain a small amount of cholesterol that can be dangerous for a normal development of an infant. Egg yolk lipids contain 10% of cholesterol (Ahn et al., 2006) and can be used in infant formulas as a source of this vital component.

As mentioned previously, egg yolk lipids can provide many important bioactive compounds for the infant, but usage of egg products for infant nutrition often raises concerns about being allergic to eggs. Egg yolk oil does not contain egg proteins and there is no risk of allergy. Extraction of egg yolk lipids is very important in terms of safety. Usage of organic solvents in extraction process may cause a risk related to solvent residues in egg yolk oil therefore the choice of solvents and technological parameters must be made with respect to safety of the infant health.

## MATERIALS AND METHODS

### Egg yolk oil extraction

Hen eggs from Lohman Brown Classic breed were purchased in local grocery store. Egg yolks from 30 eggs were separated from egg whites and homogenized.

Extraction solvents (ethanol and hexane), used in egg yolk oil extraction, were analytical grade from Sigma-Aldrich (Germany). Compressed nitrogen gas with purity 99.999 % HiQ Nitrogen 5.0 was from Linde AG (Germany).

Lipid extraction with ethanol and hexane from liquid egg yolk was made by following steps. First, polar lipids were extracted with ethanol from liquid egg yolk and then neutral lipids were extracted from precipitate with hexane (Fig. 1).

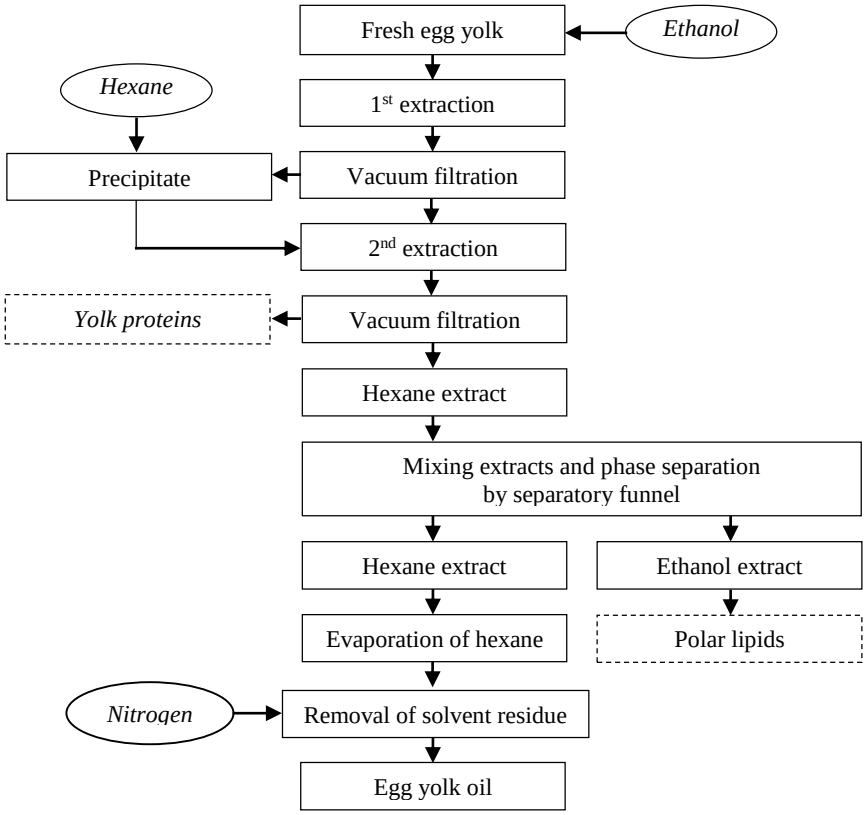


**Figure 1.** Egg yolk oil extraction process from liquid egg yolk.

For polar lipid extraction 200 g of homogenized liquid egg yolk was added to 400 ml of ethanol and stirred until egg yolk proteins denature and completely disperse. Extraction was done at 20 °C for 30 minutes. Then, the mixture was filtered by vacuum filtration, and the supernatant was collected and transferred to a separatory funnel. The precipitate containing egg yolk proteins and non-polar lipids was extracted with 400 ml hexane vigorously mixing for a 30 minutes at 20 °C using a magnet stirrer. The extract was filtered by vacuum filtration and supernatant was collected and added to the same separatory funnel. Both ethanol and hexane extracts were thoroughly but gently, to avoid emulsion formation, mixed to extract polar lipids and impurities to a polar ethanol/water phase and neutral lipids to a non-polar hexane phase. Then the mixed extracts were left for 1 hour for phase separation. Bottom ethanol/water layer, containing polar lipids and water soluble compounds was drained from the separatory funnel through the open stopcock. Hexane extract was collected in clean container. Egg yolk oil was obtained from the hexane extract by evaporation of the hexane in the rotary evaporator IKA RV 10 Control V (IKA®–Werke GmbH and Co. KG, Germany) at the temperature of 70 °C and 400 mbar pressure. After solvent evaporation in the rotary evaporator, as the last

step of the solvent removal, the pure nitrogen gas was laid through the egg oil for 10 minutes in the same rotary evaporator with the same evaporation conditions by means of a plastic tube immersed in the oil (Kovalcuks, 2014).

**Analysis**

Fatty acids

Fatty acids of egg yolk oil were determined in accordance with the standard methods ISO 12966–2:2011 and ISO 12966–1:2014, GC-FID (gas chromatography with flame ionization detector).

All chemicals and reagents used for analysis were of analytical grade and purchased from Sigma-Aldrich (Germany). 37 components FAME mix 47885-U (Supelco, Germany) was used as standard in the fatty acid content analysis. Shimadzu GC 2010 Plus gas chromatograph equipped with flame ionization detector (Shimadzu Corporation, Japan) and Nukol™ (Sigma-Aldrich, Germany) capillary GC column (30 m × 0.25 mm, $d_f$ 0.25 μm) were used. The GC analysis conditions were from ISO 12966–1:2014. Compounds were identified by comparison of the retention times of 37 components FAME mix 47885–U (Supelco, Germany).

The results were the means of three replicates with standard deviation and expressed in g 100 $g^{-1}$.

Vitamins A, D, E

Fat–soluble vitamins (α-tocopherol, cholecalciferol and retinol) content determination in egg yolk oil was made according to the Latvian standards: LVS EN 12823–1:2014; LVS EN 12821:2009 and LVS EN 12822:2014 respectively.

All solvent used in the chromatography analysis were for HPLC grade and purchased from Sigma-Aldrich (Steinheim, Germany). A-tocopherol, retinol and cholecalciferol standard solutions were purchased from Sigma-Aldrich (Germany) and then they were diluted in hexane. Vitamins were quantified using internal standard method where peak areas were identified by comparisons of retention times with those of standards used for vitamins determination.

For HPLC analysis Shimadzu Nexera X2 liquid chromatograph equipped with UV and fluorescence detector and Intersil 5 silica column (5 μm, 250 mm × 4.6 mm) was used. Temperature of the column oven was 30 °C. Mobile phase contained 3% of 1,4-dioxane in n-hexane. Flow rate of the mobile phase was 2 ml per min, and the injection volume was 20 μl. The following wavelengths for vitamin detection were used: 325 nm for retinol, 265 nm for cholecalciferol and 290 nm for α-tocopherol.

For vitamin HPLC analysis egg yolk oil sample was diluted with hexane and injected in the HPLC system. Vitamins A and E was identified by comparing of peaks with those of authentic standards and their contents were calculated on a weight basis. Peak identification and purity was operated with normal-phase chromatography system with UV (retinol) and fluorescence detections (α-tocopherols). For vitamin D analysis reverse-phase chromatography with UV detection was used after the semi-preparative clean-up procedure.

The results were the means of three replicates with standard deviation and expressed in mg $kg^{-1}$.

Lutein

1 g of egg yolk oil was accurately weighed in 50 ml glass vial and 5 ml of methanol was added. The sample with methanol was homogenized and then left overnight (16 h)

in a refrigerator at 4 °C. Then the sample was centrifuged 800 × g for 10 min. The methanol layer was transferred to a 25 ml volumetric flask. 5 ml tetrahydrofuran (THF) was added to the glass vial with sample and vial was vortexed for 30 seconds, then centrifuged at 800 × g for 5 min. The THF layer was transferred into the methanol containing volumetric flask. The sample was extracted three more times and the THF layers were combined into the volumetric flask. THF was added to make the final volume 25 ml 10 ml of extract was dried under nitrogen. The extract was resuspended in 500 ml of ethanol and vortexed for 30 seconds (Perry et al., 2009). 20 µl were injected into the HPLC system (Shimadzu Nexera X2, Japan) for lutein analysis.

All chemical for lutein analysis and solvents for the HPLC mobile phase were of HPLC grade and obtained from Sigma-Aldrich (Germany). The lutein was determined using a C30 column (3 µl, 150 mm × 4.6 mm, YMC). Lutein was monitored at 445 nm with Shimadzu SPD-M20A photodiode array detector. The mobile phase was methanol : *tert*-Butyl methyl ether : water (95 : 3 : 2, v/v, with 1.5% ammonium acetate in water) – solvent A, and methanol : *tert*-Butyl methyl ether : water (8 : 90 : 2, v/v, with 1.0% ammonium acetate in water) – solvent B. The flow rate was set at 0.4 ml $min^{-1}$ (10 °C). Gradient procedure: start at 100% solvent A; a 21-min linear gradient to 45% solvent A and 55% solvent B; 1-min hold at 45% solvent A and 55% solvent B; an 11-min linear gradient to 5% solvent A and 95% solvent B; a 4-min hold at 5% solvent A and 95% solvent B; a 2-min linear gradient back to 100% solvent A, and a 28-min hold at 100% solvent A (Perry et al., 2009). Peak identification in sample was based on comparisons with retention time and absorption spectra of known lutein standard from Sigma-Aldrich Germany. Lutein was quantified by integrating peak area in the HPLC chromatograms. The results were the means of three replicates with standard deviation and expressed in mg $kg^{-1}$.

Phospholipids

For separation of phospholipids approximately 5 g of egg yolk oil was fractionated on a 5-g column of silica gel (60–200 mesh), by sequential elution with 200 ml chloroform, 100 ml acetone, 100 ml methanol and 100 ml 0.1% phosphoric acid in methanol. The methanol fractions were combined for recovery of the total phospholipids. Solvent was removed in a rotary evaporator. The sample residue was dissolved in chloroform, washed with saturated salt solution, and then sodium bicarbonate was added until neutral. The sample was dried with sodium sulfate and filtered. The solvent was removed by rotary evaporation at room temperature. Samples were diluted with chloroform to give a 1 mg $µl^{-1}$ solution for analysis (Seri et al., 2010).

The standards of phosphatidylethanolamine and phosphatidylcholine, chloroform, methanol, ammonium hydroxide and water for the mobile phase were of HPLC grade and obtained from Sigma-Aldrich (Germany). For determination of phospholipids Shimadzu Nexera X2 with evaporating light scattering detector ELSD-LTII (Japan) was used. As the nebulizing gas, $N_2$ was used at a flow rate of 4 l $min^{-1}$, and a nebulizing temperature of 40 °C (Mounts et al., 1992).

A 125 × 4.0 mm Si – 60 column with 5 µm particle diameter (Lichrospher) was used. The elution program was a linear gradient with 80 : 19.5 : 0.5 (v/v) chloroform : methanol : ammonium hydroxide ($NH_4OH$) at t = 0 min to 60 : 34 : 5.5 : 0.5 (v/v) chloroform : methanol : water : ammonium hydroxide ($NH_4OH$) at t = 22 min and the column was allowed to equilibrate until the next injection at t = 27 min (Yalçyn et al.,

2007). The results were the means of three replicates with standard deviation and expressed in mg $100g^{-1}$.

Cholesterol

For the egg yolk and egg yolk oil cholesterol analysis the standard method of AOAC 994.10 was used with some modifications. 10 g of the egg yolk oil was transferred to a 250 ml flask, then 40 ml of ethanol-methanol-2-propanol (90 : 5 : 5) solution and 10 ml 60% KOH were added. The flask was connected to the water-cooled condenser and refluxed for 1 hour. After cooling the mixture to room temperature, 100 ml of hexane were added and the mixture was stirred for 10 min. Then 25 ml of deionized water were added and the mixture stirred for a further 15 min. After the layers were separated, hexane layer was collected in an Erlenmeyer flask. An aliquot of 25 ml from the hexane layer was evaporated in a rotary evaporator at 40 °C. The residue was dissolved in 2 ml of ethanol and 3 µl were injected into a gas chromatograph (Chung et al., 2004). For analysis Shimadzu GC 2010 Plus with flame ionization detector was used. GC conditions: column DB-5 (30 m × 0.32 mm × 0.25 µm), carrier gas: nitrogen, constant flow 0.45 ml $min^{-1}$, temperature program: 260 °C, 6 °C $min^{-1}$, 290 °C (8 min), injector: 300 °C, split 1 : 1, detector (FID): 300°C. The results were the means of three replicates with standard deviation and expressed in mg 100 $g^{-1}$.

## RESULTS AND DISCUSSION

Egg yolk oil used in this study was obtained by the solvent extraction from liquid egg yolk. Ethanol and hexane were used as less toxic solvents which are widely used in food processing. The parameters of extraction process were chosen to produce safe and qualitative product. Extraction solvent residue in the ready product was below acceptable limits (Kovalcuks, 2014). But egg yolk oil extraction from liquid egg yolk met some problems, therefore it was necessary to separate polar egg yolk lipids from non-polar lipids, so as a result non-polar lipid fraction became the egg yolk oil (Kovalcuks, 2014). Most of egg yolk bioactive compounds are non-polar (fat-soluble), but some are polar causing losses of these compounds in non-polar egg yolk oil.

Egg yolk oil bioactive compounds were compared to mature humane breast milk because colostrum and transitional milk are highly variable in terms of chemical content and their feeding to infants lasts only for few weeks.

Fat is the most variable macronutrient of mature human milk and its content varies average from 3 to 5% (Sala-Vila et al., 2005). Bioactive compound content of egg yolk oil was expressed in mg $100g^{-1}$ fat or mg $kg^{-1}$ fat, but data, found in literature, about micronutrient in human breast milk or infant formula are mostly expressed in 100 ml of milk, therefore results of human milk and infant formulas were recalculated on 100% fat content where average fat content of human breast milk and formulas was accepted as 4%.

Fatty acids

Fatty acid content of human breast milk varies by country (Lassek & Gaulin, 2014). Some authors (Ballard & Morrow, 2013; Soleimani et al., 2013) explain variety of fatty acid content by difference in mothers' diets. Fatty acid content of human breast milk of mothers from different countries is summarized in Table 1 (Saphier et al., 2013; Yuhas et al., 2006).

**Table 1.** Composition of essential fatty acid in egg yolk oil and breast milk of mothers from different countries*

| | Fatty acid content, g $100g^{-1}$ total lipids | |
|---|---|---|
| Fatty Acids | Egg yolk oil | Human breast milk** |
| Myristic acid (14:0) | 0.16 ± 0.02 | 3–12 |
| Palmitic acid (C 16:0) | 20.74 ± 0.07 | 20–23 |
| Palmitoleic acid (C16:1) | 2.08 ± 0.18 | 3–4 |
| Stearic acid (C 18:0) | 6.58 ± 0.32 | 5–7 |
| Oleic acid (C18:1n9) | 50.43 ± 0.89 | 31–38 |
| Linoleic acid (C18:2n6) | 15.57 ± 0.02 | 12–20 |
| α-linolenic acid (C18:3n3) | 1.83 ± 0.02 | 1–2 |
| Arachidonic acid (20:4n6) | 0.02 ± 0.01 | 0.4–0.5 |
| Docosahexaenoic acid (C22:6n3) | 1.17 ± 0.19 | 0.17–0.30 |

* – Israel, Australia, Canada, Chile, China, Japan, Mexico, Philippines, United Kingdom, United States; ** – fat content 4.0%.

Recommendations on fatty acid content for infant formulas usually are based on average fatty acid content of human breast milk. The best recommendation could be given if data were collected from each country and were applied for this particular country.

Saphier et al. (2013) declare that about 70% of total fatty acids in the human breast milk comprised palmitic, oleic and linoleic acids. Palmitic, oleic and linoleic acids in egg yolk oil contain more than 85% of total fatty acids. The saturated fatty acids (SFA) in breast milk are stable regardless of mother's diet (Yuhas et al., 2006). The same is observed for egg lipids, where hen's diet does not affect SFA content in eggs (González-Muñoz et al., 2009). Palmitic and stearic acid content was similar in both breast milk and egg yolk oil. Concentration of polyunsaturated fatty acids (PUFA), in opposite, depends on human and hen's diet (Saphier et al., 2013; Soleimani et al., 2013). Linoleic and α-linolenic fatty acid content was at the same level in breast milk and egg yolk oil, but oleic acid content in egg yolk oil was higher than in human milk. As PUFA content in eggs can be "designed" through hen's diet, it is possible to produce eggs which will more precisely mimic PUFA content of human breast milk (Simopoulos & Salem, 1992). Arachidonic (ARA) and docosahexaenoic (DHA) acids always raise special attention due their high biological value. ARA and DHA content in human breast milk is very low and usually do not cover infant needs of these compounds (Lassek & Gaulin, 2014). Egg yolk oil used in this study was high in DHA, but low in ARA. These two essential fatty acids come from phospholipids and their content depends on phospholipid content in egg lipids (egg yolk oil).

Fat–soluble vitamins

Fat–soluble vitamin A, D and E content in egg yolk oil, human breast milk and recommendations for the content of these vitamins in infant formulas are presented in Table 2.

Babies are born with low stores of vitamin A. Probably due to this reason colostrum contains the highest concentration of vitamin A. Later concentration of vitamin A decreases (Fujita et al., 2011). According to Lawrence & Lawrence (2016) vitamin A concentration in mature milk is on average 18.75 mg $kg^{-1}$ fat. Recommendations for infant formulas propose to use vitamin A at levels of 15–45 mg $kg^{-1}$ fat (Berthold et al., 2005). A lot of children around the world feel the lack of vitamin A that causes many

deaths of young children (WHO, 2011). But in the same time high dosages of vitamin A can be toxic. There are strong recommendations regarding upper limits of vitamin A for infant nutrition (Olson, 1989). Particular egg yolk oil contained two times lower vitamin A concentration than human breast milk, but taking into account that eggs can be enriched with vitamin A through hen diet (Jiang et al., 1994), egg yolk oil could be the good source of vitamin A for infants.

**Table 2.** Fat-soluble vitamin and lutein content in egg yolk oil, breast milk and recommendations of these compounds for infant formula

| | Concentration, mg $kg^{-1}$ fat | | |
|---|---|---|---|
| Bioactive compound | Egg Yolk Oil | Breast Milk | Recommended** |
| Vitamin A | 9.80 ± 1.18 | av. 18.75 | 15–45 |
| Vitamin D | 0.127 ± 0.015 | av. 0.015 | 0.25–1.25 |
| Vitamin E | 205.56 ± 24.67 | 25–246 | 125–1.250 |
| Lutein | 0.33 ± 0.07 | 0.375–1.425 | max 6.25 |

* – fat content 4 %
** – for infant formulas (4% fat content in ready product)

Vitamin D is a very important compound, but its content in human breast milk is very low and inadequate for normal infant development. Therefore pediatricians recommend additional peroral supplement of vitamin D for all infants till they are 1 year old (Lawrence & Lawrence, 2016). Recommendations for vitamin D in infant formulas also offer higher concentrations than naturally observed in human milk (Berthold et al., 2005). Egg yolk oil contains highly bioavailable vitamin D in concentration of 0.127 ± 0.015 mg $kg^{-1}$ fat and can be used as a natural source of vitamin D for infant nutrition.

A-tocopherol is the most active form of vitamin E therefore comparison of vitamin E content in egg yolk oil and human breast milk were made in regards of α-tocopherol. Vitamin E concentration in human breast milk depends on an individual and ranges between 25–246 mg $kg^{-1}$ fat (Lima et al., 2014). Infants from 0 to 1 year require a higher dosage of vitamin E than in later life and based on this fact recommendations for vitamin E allow to use 125–1.250 mg $kg^{-1}$ fat for infant formulas (Berthold et al., 2005).

Egg yolk oil contains vitamins A, D and E in concentrations similar to their content in mother milk and is an excellent source of these vitamins for infant nutrition.

Lutein

Lutein is a polar compound and its majority was extracted from liquid egg yolk with ethanol therefore lutein concentration in egg yolk oil (non-polar fraction of egg lipids) was at a level much lower than its possible content in egg yolk. According to Canfield et al. (2003) lutein content in human breast milk from mothers of different countries varies from 0.375–1.425 mg $kg^{-1}$ fat and is considered to be very low and insufficient for infants. Therefore recommendations of lutein content for infant formulas allow using them in concentration up to 6.25 mg $kg^{-1}$ fat (FSANZ, 2008). The difference between breast milk lutein and recommendations also lies in the fact that breast milk lutein is more bioavailable than lutein added to infant formulas (Lewis, 2014). Lutein content in human milk depends on mother's diet. Consumption of product rich in lutein increases lutein content in milk (Sherry et al., 2014). Egg yolk oil used in this study contains 0.33 ± 0.07 mg $kg^{-1}$ of lutein that was less than the lower limit of lutein in breast

milk. Purified polar fraction of egg yolk lipids can be used as a valuable source of lutein for infant nutrition.

Phospholipids

Comparing to the human breast milk egg yolk lipids contain high amount of phospholipids (PLs), approximately 30% from total lipids. Main PLs of egg yolk are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Ahn et al., 2006). Egg yolk oil contains $0.582 \pm 0.009$ mg $100g^{-1}$ of PC and $0.015 \pm 0.002$ mg $100g^{-1}$ of PE. The low concentration of PLs, the same as lutein, is connected to the polarity of these compounds. PLs are highly polar compounds and majority of PLs was extracted from egg yolk with ethanol to a polar lipid fraction. Due to the low content of PLs egg yolk oil cannot be considered as a good source of these bioactive nutrients. Relative absence of PLs negatively affects ARA content in egg yolk oil. According to Guiffrida et al. (2013) human breast milk contains 77–295 mg $100g^{-1}$ fat of PC and 80–240 mg $100g^{-1}$ fat of PE. PLs content in infant formulas is calculated taking in account desired ARA and DHA, and also choline concentration in ready product. Average dosage of total PLs can be at level of 2 g per L formula, or 5,000 mg $100g^{-1}$ fat.

Cholesterol

The average content of cholesterol in mature milk is convincingly stable at 240 mg $100g^{-1}$ fat (Lawrence & Lawrence, 2016). But according to Kamelska et al. (2012) cholesterol content in human milk is 84–492 mg $100g^{-1}$ fat. Cholesterol content in breast milk depends on an individual and changes during breast-feeding. Commercially produced infant formulas have very low cholesterol content (23–137 mg $100g^{-1}$ fat) (Kamelska et al., 2012). Cholesterol is important in proper development of nervous system, hormone and vitamin synthesis in the growing infant; therefore lack of this compound can have negative effect on his/her normal development. Egg yolk lipids naturally contain a high amount of cholesterol. Egg yolk oil presented in this study contains 3,105 mg 100 $g^{-1}$ fat of cholesterol and it is 10 times more than presented in breast milk. For a developing infant probably it can be considered as a benefit, but it must be discussed with pediatricians.

Phospholipids and cholesterol content in egg yolk oil, breast milk and infant formula is mentioned in Table 3.

**Table 3.** Phospholipids and cholesterol content in egg yolk oil, breast milk and infant formula

| | Concentration, mg $100g^{-1}$ fat | | |
|---|---|---|---|
| Bioactive compound | Egg Yolk Oil | Breast Milk* | Infant formula* |
| Phosphatidylcholine (PC) | $0.582 \pm 0.009$ | 77–295 | av. 5,000 |
| Phosphatidylethanolamine (PE) | $0.015 \pm 0.002$ | 80–240 | |
| Cholesterol | 3,105 | 84–492 | 23–137 |

* - fat content 4%

## CONCLUSIONS

- Fatty acids of egg yolk lipids more closely mimic the fatty acid composition of human breast milk. Moreover, fatty acid profile of egg yolk lipids can be affected by a hen diet which allows providing the product for specific customer needs. Fatty acid profile of egg yolk oil used in this research was similar to breast milk in terms of palmitic, stearic, linoleic and α–linolenic acids. DHA content was

$1.17 \pm 0.19$ mg $100g^{-1}$ being in compliance with infant needs, but content of ARA was much lower than found in human breast milk. Content of ARA in egg yolk oil was $0.02 \pm 0.01$ mg $100g^{-1}$ in comparison with 0.4–0.5 mg 100 $g^{-1}$ fat in human breast milk.

- Egg yolk oil contains essential fat-soluble vitamins such as vitamins A, D and E. There is no doubt about their importance for a developing organism. But concentration of these vitamins in infant food is of utmost importance. Vitamin A concentration in particular egg yolk oil was twice lower ($9.80 \pm 1.18$ mg $kg^{-1}$) than observed in human milk, but it can be easily improved by enrichment of eggs with vitamin A through hens' feed. Vitamin D concentration in egg yolk oil was in the middle between its content in breast milk and recommended by pediatricians for infant nutrition. Usually the lack of vitamin D in infant nutrition is compensated by additional oral supplement via food additive (vitamin D drops). But more important is to receive vitamin D in natural and highly bioavailable form. Egg yolk oil used in this research can provide $0.127 \pm 0.015$ mg $kg^{-1}$ of natural vitamin D. Vitamin E content in egg yolk oil was $205.56 \pm 24.67$ mg $kg^{-1}$. This natural antioxidant content in human breast milk can be found in concentration 25–246 mg $kg^{-1}$ therefore egg yolk oil can fully compensate infant needs of this vitamin.
- Breast milk contains a high amount (84–492 mg 100 $g^{-1}$ fat) of cholesterol, while infant formulas contain much less (23–137 mg $100g^{-1}$ fat). Egg yolk, being an animal source product, naturally contains a high amount of cholesterol that is perceived as a disadvantage for adult nutrition, but not for infants. High content of cholesterol (3,105 mg 100 $g^{-1}$) in egg yolk oil can be considered as a benefit in terms of infant nutrition, since it is responsible for brain and central nervous system development of an infant.
- Lutein content in egg yolk oil, due to specifics of extraction process, was low ($0.33 \pm 0.07$ mg $kg^{-1}$) comparing to its content in human milk (0.375–1.425 mg $100g^{-1}$ fat). Lutein is a polar compound and in two solvent system, containing ethanol and hexane, majority of lutein was extracted into polar ethanol phase. Bioavailability of egg yolk lutein is much higher than from other sources therefore addition of egg yolk lutein in infant formulas has an important benefit for infant health and development.
- The main egg yolk oil phospholipids are phosphatidylcholine and phosphatidylethanolamine whose summary content in extracted egg yolk oil was 0.597 mg 100 $g^{-1}$. The same as lutein, phospholipids are polar compounds and their content in egg yolk oil depends on solvents used for extraction process. Phospholipids provide infants with ARA, DHA and choline, essential nutrients for normal infant development, therefore low content of phospholipids in egg yolk oil must be compensated by other ingredients.
- Purified polar fraction of egg yolk lipids, containing polar bioactive compounds such as lutein and phospholipids can be used as a separate additive for infant formulas increasing their biological value.
- High content of bioactive compounds of egg yolk oil makes it an ideal ingredient for infant formulas, supplying infants with PUFA, fat-soluble vitamins and cholesterol.

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# Total phenols and antioxidant capacity of hull-less barley and hull-less oats

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**Abstract.** Grain products are the main source of carbohydrates but they also contain other bioactive substances such as phenolic compounds. Content of phenolic compounds differ among cereal types, varieties, and farming methods. The aim of the current study was to assess total phenolic content and radical scavenging activity in different oats and barley varieties compared to hulled ones. In the experiment hull-less varieties / lines were analysed: three barley (line 'GN 03386', from Norway and 'Kornelija', 'Irbe' from Latvia) and three oats varieties ('Bikini', 'Nudist', from Norway and 'Stendes Emilija,' from Latvia). One hulled variety of barley and oats from each country was included in the experiment for comparison. For the isolation of phenolic compounds ultrasound assisted extraction was used. For all extracts the total phenol content and DPPH, $ABTS^{+}$ radical scavenging activity were determined spectrophotometrically. Overall, the highest content of total phenols was detected in hull-less barley samples. The barley variety with the highest content was line 'GN 03386,' followed by varieties 'Kornelija', 'Irbe,' and hulled Norwegian barley variety 'Tyra'. High DPPH and $ABTS^{+}$ radical scavenging activity was recorded in barley line 'GN 03386'. Generally, there was strong correlation between total phenol content and $ABTS^{\bullet+}$radical scavenging activity and moderate correlation between total phenol content and DPPH radical scavenging activity. In conclusion, the barley varieties had generally higher content of bioactive substances than oats and the barley line 'GN 03386' seems to be one of the best.

**Key words:** hull-less oats, hull-less barley, total phenols, antioxidant.

## INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the ancient grain cultures, which is widely used as fodder and food, especially in malt production. For many years, pearled grains have been among of the most popular barley products. Researchers have investigated different approaches for using barley in bread industry – sourdough (Mariotti et al., 2014), rye bread (Pejcz et al., 2015), and wheat bread (Rieder et al., 2012) production. Hull-less barley has been confirmed to be a good source of both insoluble and soluble fractions of dietary fibre and other bioactive compounds (Blandino et al., 2015) that

makes beneficial effects on human health (Tong et al., 2015). In Latvia, breeding programs focus on hull-less barley morphological characteristics, agronomical, physical and chemical parameters (Zute et al., 2012; Bleidere et al., 2013a; 2013b; Šterna et al., 2015) and potential use of hull-less barley in beer production (Dabina-Bicka et al., 2011).

Oats (*Avena sativa* L.) is highly recognized for its high energy and nutritional value due to high content of proteins and lipids. Oats are also a good source of soluble fibre, essential amino acids, unsaturated fatty acids, vitamins, minerals, and phytochemicals (Jones, 2003; Arendt & Zannini, 2013; Vilmane et al., 2015). Chen et al. (2015) reported that oats contain abundant antioxidant compounds, including tocols (Shewry et al., 2008), sterols (Peterson, 2001; Shewry et al., 2008), phenolic compounds (Shewry et al., 2008) and phytic acid (Peterson, 2001). All over the world hull-less oats are mainly used for fodder. However, the grain chemical content and nutritional value has aroused interest for their use in human nutrition (Behall & Hallfrisch, 2011; Tiwari & Cummins, 2012; Redaelli et al., 2013; Vilmane et al., 2015). Compared with hulled oats grain, hull-less oats grain contains less fibre, more protein and lipids, and has higher energy value (Givens et al., 2004; Biel et al., 2009).

Most common hulled varieties of barley and oats require mechanical removal of the tenacious hull covering the grain. This process also removes most of the bran layer and germ thus resulting in loss of valuable components. Therefore recently new hull-less barley and hull-less oats varieties have been developed in order to ensure both high productivity level, along with straw strength, disease resistance and increased grain quality (Bleidere et al., 2014).

According to the review of Acosta-Estrada et al. (2014) most of the beneficial properties of grains have been attributed to bioactive non-nutritional chemical compounds commonly named phytochemicals. Among these, phenolic compounds have been extensively studied due to their diverse health benefits as antioxidants, and for preventing chronic inflammation, cardiovascular diseases, cancer and diabetes. This effect seems to be partly due to phytochemicals that combat oxidative stress (Masisi et al., 2016). With increased consumer knowledge on the health benefits provided by soluble dietary fibre and other grain constituents, barley and oats are becoming more attractive for researchers and producers.

Phenolic compounds are considered as a major group in grains that contribute to the antioxidant activity of cereal. These molecules are secondary metabolites of plants possessing possible positive physiological effects (Peng et al., 2015). Dietary antioxidants play a significant role in human health by prevention of radical damage to biomolecules such as DNA, RNA, proteins, and cellular organelles. The antioxidant activity of polyphenols has been mainly related to their redox properties, which can play an important role in neutralizing free radical and quenching oxygen or decomposing peroxides (Kahkonen et al., 1999).

There are various methods suitable for evaluation of phenolic content and antioxidative capacity in plants, foods and ingredients (Moon & Shibamoto, 2009; Kammerer et al., 2011). The initial analytical approach consists of using non-specific methods in order to determine the overall content of phenolic compounds, usually expressed as an index such as gallic acid, chlorogenic acid or catechin equivalent. A more detailed approach using chromatography can specifically quantify certain compounds of interest. Most phenolic compounds in cereal-based matrices are in the insoluble bound forms (Acosta-Estrada et al., 2014). Phenolic compounds in oats and

other grains mainly exist in bound forms and are typical components of complex structures such as lignins, hydrolysable tannins, and organic acids (Alrahmany & Tsopmo, 2012).

Bleidere et al. (2014) found significant differences among cultivars in antioxidant activities and total phenolic contents (TPC). Oats contain tocols, phenolic acids, aventhramides, flavonoids and sterols (Bryngelsson et al., 2002; Dimberg et al., 2005). These groups of compounds are located mainly in the outer layers of the kernel (Pecio et al., 2013). For barley, Dvorakova et al. (2008) reported the phenolic acids such as the hydroxybenzoic (protocatechuic, gallic, vanillic, and syringic) and the hydroxycinnamic acids (caffeic, sinapinic, p-coumaric, and ferulic). Ferulic acid was clearly the most abundant phenolic compound found in the bound form in barley.

Despite the fact that many studies have been conducted on bioactive compounds in hull-less oats and barley, the results are contradictory. Therefore, the aim of the current study was to assess total phenolic content and radical scavenging activity in different hull-less oats and barley varieties comparing to hulled ones.

## MATERIALS AND METHODS

Chemical analyses were performed at scientific laboratories of the Latvia University of Agriculture, Faculty of Food Technology. The phenolic compounds, DPPH, and ABTS radical scavenging activity were determined for oats (hulled and hull-less) and barley (hulled and hull-less).

### Grain materials

Grain samples were selected from seed material of different cereal cultivars grown in Latvia and Norway.

In the study three hull-less Latvian and Norwegian barley varieties / lines (line 'GN 03386', from Norway and 'Kornelija', 'Irbe' from Latvia) were tested along with hulled barley varieties 'Rubiola' (Latvia) and 'Tyra' (Norway). Similarly, three hull-less oats varieties ('Bikini', 'Nudist', from Norway and 'Stendes Emilija' from Latvia), as well as hulled oat varieties 'Laima' (Latvia) and 'Odal' (Norway) were included in the study. The moisture content of grains at analysing stage was 12.0–12.9%.

### Chemical analysis

*Chemicals*

Gallic acid (97.5%), Folin-Ciocalteu phenol reagent, 2,2-diphenyl-1-picrylhydraziyl (DPPH$^{\cdot}$) (99%), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS$^{\cdot+}$) (98%), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (97%) were purchased from Sigma-Aldrich (Switzerland). All other chemicals used in for analyses were obtained from Acros Organic (USA).

*Extraction of phenolic compounds from grains*

The homogenized grain samples (2.0 g) were extracted with ethanol/acetone/water (7/7/6 v/v/v) solution in an ultrasonic bath YJ5120-1 (Oubo Dental, USA) at 35 kHz for 10 minutes at 20 ± 1 °C temperature. The extracts were then centrifuged in a centrifuge CM-6MT (Elmi Ltd., Latvia) at 3,500 min$^{-1}$ for 5 min (RCF 2300). Residues were re-extracted using the same procedure. Ratio of sample versus solvent was 1:10. Triplicate extraction process was done.

*Determination of total phenolic compounds*

The TPC of the grain extracts was determined according to the Folin-Ciocalteu spectrophotometric method (Singleton et al., 1999). To 0.5 mL of extract 2.5 mL of Folin-Ciocalteu reagent (diluted 10 times with water) was added and, after 3 min 2 mL of sodium carbonate water solution ($Na_2CO_3$) (75 g $L^{-1}$) was added. Then sample was mixed. The control sample contained all the reaction reagents except the extract. After 30 minutes of incubation at room temperature, the absorbance was measured at 765 nm. The results were calculated using standard curve of gallic acid with the range of the standard of 10 mg to 80 mg GAE $L^{-1}$. Total phenols were expressed as gallic acid equivalents (GAE) per 100 g dry weight (DW) of the samples.

*Determination of DPPH˙ radical scavenging activity*

Antioxidant activity of the grain extracts was measured on the basis of scavenging activities of the stable 2.2-diphenyl-1-picrylhydraziyl (DPPH˙) radical as outlined by Yu et al. (2003). The antioxidant reaction was initiated by transferring 0.5 m L of plant extract into a sample cavity containing 3.5 mL of freshly prepared DPPH˙ methanol solution (0.004 g DPPH˙ to 100 mL methanol). The absorbance was measured at 517 nm, after 30 min of incubation in the dark at room temperature. The radical scavenging capacity was expressed as Trolox mM equivalents (TE) 100 $g^{-1}$ DW of the samples. The standard curve was prepared for the concentrations of solutions between 5–10 μM Trolox.

*Determination of $ABTS^{\cdot+}$ radical scavenging activity*

The radical scavenging capacity of extract was measured also by $ABTS^{\bullet+}$ radical cation assay (Re et al., 1999). Firstly, phosphate buffered saline (PBS) were prepared by dissolving 8.18 g sodium chloride (NaCl), 0.27 g potassium dihydrogen phosphate ($KH_2PO_4$), 1.42 g sodium phosphate dibasic ($Na_2HPO_4$), and 0.15 g potassium chloride (KCl) in 1 L of ultra-pure water. A stock solution of ABTS (2 mM) was prepared in 50 mL of PBS. If the pH was lower than 7.4, it was adjusted with sodium hydroxide (NaOH). Ultra-pure water was used to prepare 70 mM solution of potassium persulfate ($K_2S_2O_8$). $ABTS^{\bullet+}$ radical cation was produced by reacting 50 mL of ABTS stock solution with 0.2 mL of $K_2S_2O_8$ solution and allowing the mixture to stand in the dark at room temperature for 15–16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the assessment of extracts, the $ABTS^{\bullet+}$ solution was diluted with PBS to obtain the absorbance of 0.800±0.030 at 734 nm. Five mL of $ABTS^{\cdot+}$ solution were mixed with 0.05 mL of extract. The absorbance was read at ambient temperature after 10 min. PBS solution was used as a blank sample. The radical scavenging capacity was expressed as Trolox mM equivalents (TE) 100 $g^{-1}$ DW of the samples. The standard curve was prepared for the concentrations of solutions between 2–10 μM Trolox.

*Statistical analysis*

Experimental results presented are means of three parallel measurements and were analyzed by Microsoft Excel 2010 and SPSS 17.00. Analysis of variance (ANOVA) and Tukey test were used to determine differences among samples. A linear correlation analysis was performed in order to determine relationship between TPC, antioxidant activity such as DPPH˙, and $ABTS^{\bullet+}$ radical scavenging activity. Differences were considered as significant at $P < 0.05$.

## RESULTS AND DISCUSSION

### Total phenolic compounds (TPC)

The TPC in oats ranged from 179 to 221 mg GAE $g^{-1}$ DW (Fig. 1A). Difference among varieties was significant and this may indicate variation in genetic background, growing conditions, agrotechnology, and other factors among cultivars. In our study it is difficult to consider which factors have affected TPC content in grains because of the limited information about growing conditions. Chu et al. (2013) reported lower TPC values – in oats ranged from 57 mg to 94 mg 100 $g^{-1}$. On the other hand total phenolic content in the oats studied by Brindzová et al. (2008) had higher values than in our study and differed significantly between the varieties ranging from 239 to 662 μg GAE $g^{-1}$ DW. Results of our study revealed that the highest TPC content was in oats varieties 'Stendes Emilija', 'Odal,' and 'Laima'. Thus indicating that influence of variety is more significant than grain type – hull-less or hulled. Similar results reported Bleidere et al. who did not find notable difference between hulled and hull-less standard varieties in content of total phenolic compounds in grain (Bleidere et al., 2013a).

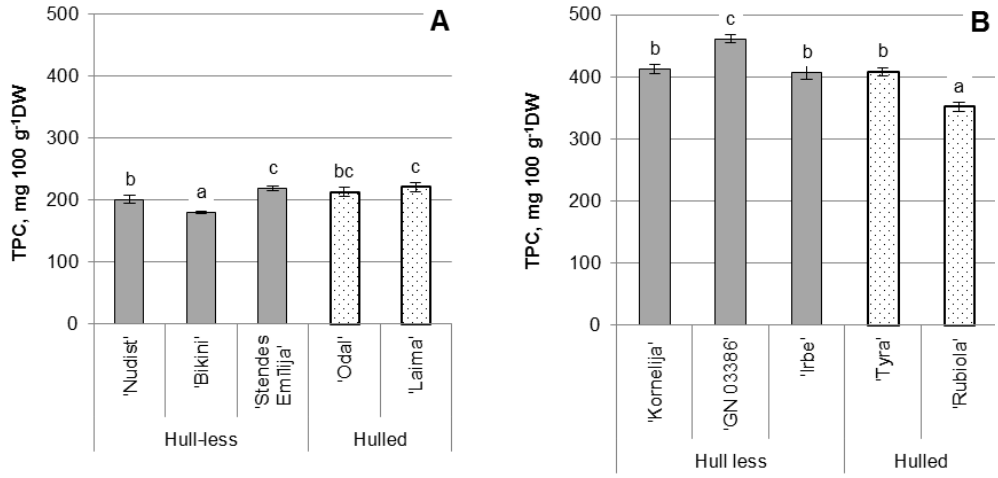


**Figure 1.** Total phenolic compounds in oats (A) and barley (B) samples. Note: the values marked with different letters for each cereal type represent significant differences between values ($P < 0.05$).

The TPC for barley ranged from 351 to 460 mg GAE 100 $g^{-1}$ DW (Fig. 1B). Bleidere et al. (2013a) reported lower TPC with high variation: from 143.6 to 262.1 mg GAE 100 $g^{-1}$ with coefficient of variation 13.4%. In dehulled highland barley from China phenolic content ranged from 167.9 ± 12.1 to 282.0 ± 5.5 mg 1grain (Zhu et al., 2015). Also for barley, the variety is the most significant factor influencing TPC not the type – hull-less or hulled. Similar results reported Bleidere et al. – that there was also no notable difference between hulled and hull-less standard varieties in content of total phenolic compounds in grain (Bleidere et al., 2013a). In hull-less barley varieties grown in India TPC varied significantly within cultivars and ranged between 278 to

338 mg 100 $g^{-1}$ (Moza & Gujral, 2016). Comparing both cereal types (Fig. 1A and 1B) it can be clearly seen that barley generally has significantly higher TPC.

Also DPPH scavenging activity was significantly influenced by variety not the grain type – hull-less or hulled (Fig. 2). Among oats varieties significantly lower DPPH scavenging activity was found only for variety 'Bikini' (Fig. 2A). From barley samples significantly higher activity was in line 'GN 03386,' followed by 'Rubiola' (Fig. 2B). Differences between oats and barley in DPPH scavenging activity were not significant.

The main phenolic classes in oats include phenolic acids, flavonoids and a unique group avenanthramides and several studies showed strong antioxidant capacity of this specific group (Yang et al., 2014), that could explain high DPPH radical scavenging activity, even if TPC is significantly lower, compared to barley. Also opposite results are reported that avenanthramide levels did not correlate with the observed antioxidant capacities, suggesting that other phytochemicals may contribute significantly or synergistically to the wide free radical-scavenging capacities of oats (Chu et al., 2013). Oats contain bioactive peptide lunasin that could also demonstrate antioxidant properties (Nakurte et al., 2013). Tocopherols and tocotrienols found in oats are natural antioxidants, but there was not found correlation between their content and activity (Chu et al., 2013). Oats antioxidant activity could be explained by active components and synergistic effects and interactions among the various antioxidants that give rise to a net antioxidant capacity in different oats varieties (Chu et al., 2013).

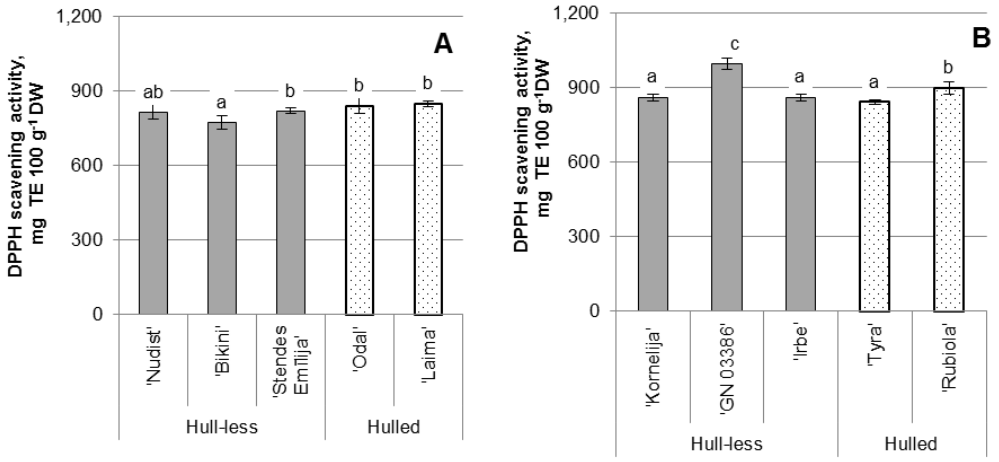


**Figure 2.** DPPH scavenging activity of oats (A) and barley (B) samples. Note: the values marked with different letters for each cereal type represent significant differences between values ($P < 0.05$).

The abundant content of phenolic compounds in barley reveals that it may serve as an excellent dietary source of natural antioxidants with antiradical and antiproliferative potentials for disease prevention and health promotion (Zhao et al., 2008). Žilić et al. (2011) reported that among the small grain species, hull-less barley had the highest reducing power, contained the most active scavengers of free radicals.

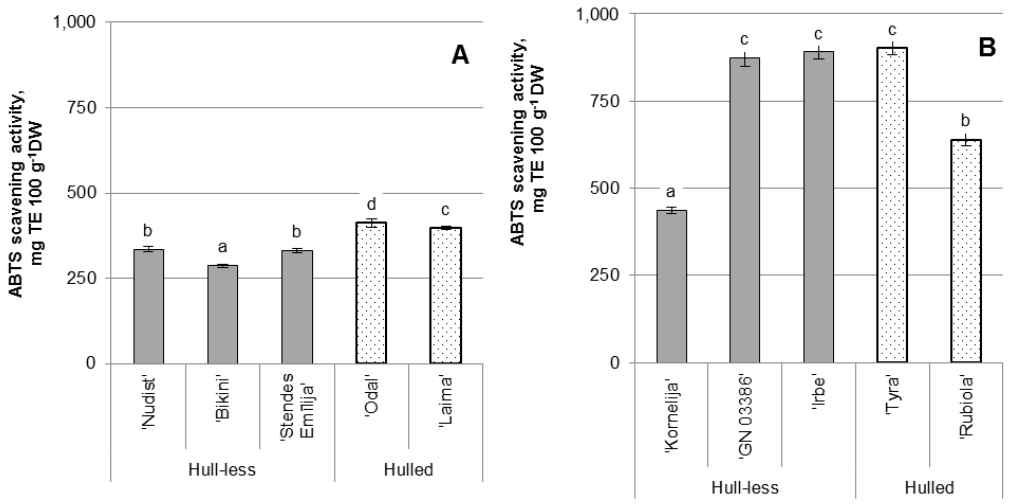


**Figure 3.** ABTS scavenging activity of oats (A) and barley (B) samples.Note: the values marked with different letters for each cereal type represent significant differences between values ($P < 0.05$).

In oats the highest ABTS scavenging activity was in variety ‘Odal’, and generally both hulled oats species demonstrated significantly higher activity (Fig. 3A). In barley the highest activity was in varieties ‘Irbe’, ‘Tyra,’ and in line GN 00386 (Fig. 3B). The ABTS scavenging activity also depends on variety and not of grain type. Differences between oats and barley in ABTS scavenging activity were significant, with the highest values for barley. Results showed that ABTS assay is more specific for analysed samples and it is possible to see differences between them, comparing to DPPH radical assay. Different ABTS results from the DPPH could be explained with different reaction mechanism. ABTS + radical is stable and is much more active than DPPH˙ radical. ABTS radical cation reactions with antioxidant is faster than the millisecond (Naik et al., 2003). ABTS reacts with most of the antioxidants, it does not affected by the ionic strength and is used to determine both hydrophilic and hydrophobic antioxidant activity (Martysiak-Żurowska & Wenta, 2012). Also the results of a variety of foods suggest that ABTS assay better reflects the antioxidant contents than DPPH assay and the correlation between antioxidant capacities detected by ABTS and DPPH assays was strong in fruits and beverages, but lower in vegetables. Most vegetables analyzed showed much lower antioxidant capacities as measured by DPPH assay relative to ABTS assay (Floegel et al., 2011).

**Relationship between phenolic compounds and antioxidant capacity**

Phenolic compounds are one of the compounds group posing radical scavenging activity. Pearson’s coefficient between TPC and DPPH scavenging activity was strong ($r = 0.74$) but between TPC and ABTS scavenging activity it was very strong ($r = 0.86$). Dordevic et al. (2010) did not find correlation between TPC and DPPH scavenging activity in the grains. Also Brand-Williams et al. (1995) reported similar results. In contrast, significant ($P < 0.05$) positive correlation between radical scavenging activity

and total phenolic content ($r$ = 0.519) was obtained by Bleidere et al. (2013a) and Zhao et al. (2008) in spring barley.

Very strong correlation between TPC and ABTS scavenging activity ($r$ = 0.971) was reported for commercial canola meal (Hassas-Roudsari et al., 2009) and durum ($r$ = 0.950) (Žilić et al., 2012). Whereas Italian researchers analysing whole grain durum wheat (*T. durum* Desf.) determined strong correlation ($r$ = 0.663) (Laus et al., 2012).

## CONCLUSIONS

The present study determined TPC and antioxidant activity in grains of five oats and five barley varieties from Latvia and Norway. For oats and for barley, TPC and antioxidant activity was significantly influenced by cultivar variety. The type of grain-hull-less or hulled had no effect on analysed compounds.

All barley varieties had higher TPC and ABTS scavenging activity comparing to the oats varieties. The highest activity was detected in hull-less barley line 'GN 03386'. Impossible was to select the best oats variety with the highest parameters, but significantly lower TPC and antioxidant activity was found in hull-less oats variety 'Bikini'. Bioactive compounds should be taken into consideration developing new functional products.

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# Effects of germination on total phenolic compounds and radical scavenging activity in hull-less spring cereals and triticale

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**Abstract.** The aim of the current research was to evaluate changes in the content of total phenolic compounds and radical scavenging activity at different germination stages of triticale, hull-less barley, hull-less oats, wheat, and rye. Grain germination was performed for 12, 24, 36 and 48 h at controlled conditions. Ultrasound assisted extraction was used for isolation of total phenolic compounds. For all extracts the total phenolic compounds content, DPPH radical and $ABTS^+$ radical scavenging activity were determined spectrophotometrically. The results of the experiments demonstrated that the highest content of total phenolic compounds and the highest antiradical activity was determined in hull-less barley samples. In all studied grains the content of phenolic compounds increased significantly during soaking and germination process. DPPH radical scavenging activity during germination increased. $ABTS^+$ radical scavenging activity also increased after soaking process and dynamics were cereal type dependent. Pearson's coefficients between the phenolic compound levels and antioxidant activity taking into account all obtained results were high. Very strong positive correlations between the content of phenolic compounds and antioxidant activity were determined for triticale during germination. Also antioxidant activity determined by both tests correlated with the highest results for barley and oats. The highest content of total phenolic compounds determines the optimum duration of germination to be 24 hours, except rye samples where the highest value was reached only after 48 hours of germination. Shorter time was required to reach the highest values of DPPH radical activity – for wheat rye, and triticale, namely 12 hours.

**Key words:** cereals, germination, total phenolic compounds, radical scavenging activity.

## INTRODUCTION

Grains and related processed products are consumed globally as important energy sources. Grain-based foods provide the majority of the carbohydrates, some proteins, lipids, dietary fiber, and other micronutrients in many diets (Luthria et al., 2015). Human diet includes the most important grains – wheat, rice, and corn, which are supplemented by other minor grains – oats, barley, rye, triticale, sorghum, millet, and buckwheat. It is well documented that germinated cereals have a greater nutritive and physiological value than un-germinated cereal and pseudocereal grains and their products (Prodanov et al., 1997; Price, 1988; Rozan et al., 1999; Rozan et al., 2000; Donkor et al., 2012). These

modified (germinated) grains, with increased levels of bioactive compounds, may have capacity in combating burning health issues among the obese and diabetics, as well as the potential to reduce the risk of colon cancer (Donkor et al., 2012).

The bioactive phytochemicals in wheat (*Triticum aestivum* L.) can be broadly subdivided into the following categories: phenolic acids, carotenoids, tocopherols, alkylresorcinols, and other miscellaneous compounds (sterols, steryl ferulates, benzoxazinoids and lignans) (Luthria et al., 2015). Wheat is commonly processed prior to consumption. Grain producers, processors, consumers, health, and nutritional professionals are interested in investigating the effect of processing on bioactive phytochemicals present in wheat (Wang et al., 2014). Rye (*Secale cereale* L.) has traditionally had an important role in the daily diet especially in Northern and Eastern Europe, and in addition to dietary fibre, rye grain is a good source of various phytochemicals (Pihlava et al., 2015) and be exploited more efficiently in new types of cereal products due to its positive health effects. Nowadays, its use is limited mainly due to the problems arising from its flavour; not all European consumers are familiar with the somewhat foreign, rye-like flavour, perceived as bitter and intense (Heiniö et al., 2003). Among the phytochemicals in rye, phenolic acids, alkylresorcinols and lignans, in particular, have gained a special focus (Bondia-Pons et al., 2009). Triticale is a hybrid of wheat (*Triticum*) and rye (*Secale*). Triticale (X *Triticosecale* Whittmack) is a potential alternative to wheat in processed flour products such as bread, flat bread, cakes or pasta. Triticale is rich in phenolics and dietary fibres consisting of both soluble and insoluble fibres (Agil & Hosseinian, 2014). Barley (*Hordeum vulgare* L.) has been intensively investigated in respect to its food, feed and industrial applications. Of the various barley cultivars, hull-less barley has recently been receiving considerable attention concerning the development of functional food, as it is an excellent source of both soluble and insoluble fibre. Hull-less (or 'naked') barley *(Hordeum vulgare* L. var. *nudum* Hook. f.) is a form of domesticated barley, in which, unlike hulled barleys but similarly to wheat, the lemma and palea (hull) are non-adherent to the caryopsis (Blandino et al., 2015). The advantage of hull-less barley compared to hulled barley in food uses is that pearling is not needed, so that the outer part of the endosperm, the aleurone, which contains proteins with essential amino acids and vitamins, is retained, as well as other bioactive compounds (Andersson et al., 2004). The nutritional improvement of hull-less oat relates to relatively high, energy rich oil content along with high protein content with a good balance of the amino-acids lysine, methionine and cysteine (Stroh et al., 2006). The most important bioactive compounds of oats (*Avena sativa* L.) are phenolic compounds. Some oat phenolics have great potential as nutraceuticals while some others are powerful antioxidants (Kilci & Gocmen, 2014). Oats contain antioxidants, and oat lipids are stable in mature, undamaged grains and in sufficiently heat-treated oat products (Molteberg et al., 1995).

Phenolic compounds are considered as a major group of compounds that contribute to the antioxidant activity of cereal. Moreover, upon germination the concentrations of these antioxidants increase. These molecules are secondary metabolites of plants possessing some positive physiological effects (Peng et al., 2015). Dietary antioxidants may play a significant role in human health preventing radical damage to biomolecules such as DNA, RNA, proteins, and cellular organelles. Therefore, there is increasing interest in identifying and assessing commonly consumed foods. The antioxidant activity of polyphenols has been mainly related to their redox properties, which can play an

important role in neutralizing free radical and quenching oxygen or decomposing peroxides (Kahkonen et al., 1999). Phenolic compounds are mainly concentrated in the bran fraction and covalently bound to indigestible polysaccharides (Wang et al., 2014). Due to hindrance by cereal matrices, most of the bound phenolic compounds are not accessible to attack by enzymes in the human gastrointestinal tract, leading to a low bioavailability. However, bioavailability of the bound phenolic compounds could be enhanced by increasing their accessibility primarily through particle size reduction, structural breakdown of cereal matrices, and their liberation from cereal matrices using suitable processing technologies (Wang et al., 2014).

Germination, a complex process causing physical, chemical and structural changes in grains, has been identified as an inexpensive and effective technology for improving cereal quality (Wu et al., 2013). The germination process is characterized by the growth of the embryo of the grain, manifested by the rootlets growth and increase in length of the shoot (acrospire), with the concomitant modification of the contents of the endosperm (Guido & Moreira, 2013). Factors influencing the germination include intrinsic parameters, such as cultivar or variety, and storage conditions, as well as external factors, such as temperature, humidity, presence of oxygen or air, light exposure and pH for germination (Cho & Lim, 2016).

During the process of germination significant changes in the biochemical, nutritional and sensory characteristics of cereals occur due to degradation of reserve materials as used for respiration and synthesis of new cell constituents for developing embryo in the seed. As compared to un-germinated seed, germinated seeds contain high protein, low unsaturated fatty acids, low carbohydrate, mineral content and vitamins (Narsih et al., 2012; Sharma et al., 2016). The phenols synthesised during seed germination could help in order obtain enhanced levels of phenols and antioxidant activity resulting in their improved nutraceutical properties (Cevallos-Casals & Cisneros-Zevallos, 2010). Intense biochemical processes occur during the grain activation (in the first stage of germination), as a result the content of vitamins B2, E and niacin, total sugar, dietary fibre and glucosamine increase; vitamin C is synthesized, and the content of essential amino acids is increased during the process of protein hydrolysis (Rakcejeva, 2007).

At the initial germination stages phenolics may serve as radical scavengers or antioxidants, while later they could become part of the structural framework of the growing plant and lose some of their antioxidant efficiency (Cevallos-Casals & Cisneros-Zevallos, 2010). Several studies suggested that germination significantly improved the functional (Singkhornart et al., 2014) and sensory (Ohtsubo et al., 2005; Singkhornart et al., 2014) properties of cereals and they can be used as a new approach to further developmentof a potential cereal products for human consumption.

The aim of the current research was to evaluate changes in the content of total phenolic compounds and radical scavenging activity at different germination stages of triticale, hull-less barley, hull-less oats, wheat, and rye.

## MATERIALS AND METHODS

### Plant material

The grains of conventionally grown hull-less barley (cv ‘Irbe’), hull-less oat (cv ‘Lizete’), rye (cv ‘Kaupo’), and wheat (cv ‘Elvis’) at State Priekuli Plant Breeding

Institute in Latvia and triticale (cv 'Tulus') cultivated at Norwegian Institute for Agricultural and Environmental Research (Norway) were tested. The experiments were carried out at the scientific laboratories of the Faculty of Food Technology at Latvia University of Agriculture.

**Germination and sample preparation**

The grains of all cultivars were cleaned, washed and soaked in water at the ratio of 1 : 2 (grains to water) for 24 ± 1 h at 22 ± 2 °C. After soaking, water was drained and grains were placed for germination in the climatic chamber ICH110 (Memmert, Germany) at controlled temperature (35 ± 1 °C) with relative humidity (RH) 95 ± 2% in the dark. Duration of the germination was 12, 24, 36, and 48 hours. Thereafter, the germinated grains were ground in a laboratory mill KN 195 Knifetec™ (Foss, Denmark) and analysed. Moisture content of germinated cereals was determined according to the AACC method 44-15A, which includes moisture removal at 135 °C for 90 min (AACC, 2000).

As a control grain sample un-soaked and un-germinated grains were tested.

**Chemical analysis**

*Extraction of phenolic compounds from grains*

The homogenized grain samples (2.0 g) were extracted with ethanol/acetone/water (7/7/6 v/v/v) solution in an ultrasonic bath YJ5120-1 (Oubo Dental, USA) at 35 kHz for 10 minutes at 20 ± 1 °C temperature. The extracts were then centrifuged in a centrifuge CM-6MT (Elmi Ltd., Latvia) at 3,500 $min^{-1}$ for 5 min. Residues were re-extracted using the same procedure. The extraction process was done in triplicate.

*Determination of total phenolic compounds*

The total phenolic content (TPC) of the grain extracts was determined according to the Folin-Ciocalteu spectrophotometric method (Singleton et al., 1999). To 0.5 ml of extract 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) was added and, after 3 minutes 2 ml of sodium carbonate water solution (Na2CO3) (75 g $l^{-1}$) was added. The sample was mixed. After 30 minutes of incubation at room temperature, the absorbance was measured at 765 nm. Total phenols were expressed as gallic acid equivalents (GAE) 100 $g^{-1}$ dry weight (DW) of the samples. The absorbance was measured at 765 nm and total phenols were expressed as the gallic acid equivalents (GAE) 100 $g^{-1}$ dry weight (DW) of grain material.

*Determination of antioxidant activity*

Antioxidant activity of the grainextracts was measured on the basis of scavenging activities of the stable 2,2-diphenyl-1-picrylhydraziyl (DPPH) radical as outlined by Yu et al. (2003). The absorbance was measured at 517 nm. The radical scavenging activity of extract was also measured by 2,2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic) acid ($ABTS^{+}$) radical cation assay (Re et al., 1999). For the assessment of extracts, the $ABTS^{\bullet+}$ solution was diluted with a phosphatebuffersolution to obtain the absorbance of 0.800 ± 0.030 at 734 nm. The radical scavenging activity was expressed as Trolox equivalents (TE) 100 $g^{-1}$ DW of plant material.

*Statistical analysis*

Experimental results were means of three replications and were analyzed by Microsoft Excel 2010 and SPSS 17.00. Analysis of variance (ANOVA) and Tukey's and Pearson`s tests were used to determine differences among samples. A linear correlation analysis was performed in order to determine relationship between TPC, antioxidant activity such as DPPH$^{\bullet}$, ABTS$^{\bullet+}$ scavenging activity. Differences were considered as significant at $P < 0.05$.

## RESULTS AND DISCUSSION

### Total phenolic content

Soaking and germination process influenced significantly the total phenolic content (TPC) of all tested grain types (Fig. 1). In soaked grains TPC generally increased, with exception of oat grain where significant decrease was observed. The TPC did not change significantly after 12 h of germination in wheat and barley samples, decreased in rye and triticale and increased in oat samples. Prolonged germination time mainly increase the content of TPC. Significant decrease occurred in TPC content after 48 hours in barley and wheat samples. Increase of TPC most probably occurs due to distribution of phenolic acids during germination process by starch enzymatic hydrolyses (Maillard et al., 1996; Tian et al., 2004). Chen et al. (2016) also studied changes in the total phenolic content of canary seeds and reported a general trend – germinated seeds are reach in total phenolic then raw and soaked seeds (Chen et al., 2016). Another study revealed that in different seeds accumulated phenolics and antioxidant activity showed the general trend distribution of 7 day sprouts > raw seeds > steeped seeds (Cevallos-Casals & Cisneros-Zevallos, 2010).

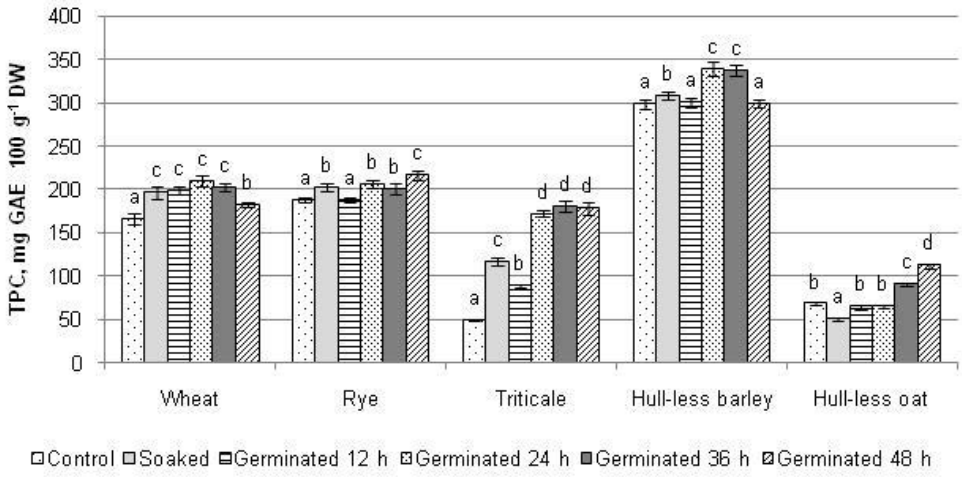


**Figure 1.** Dynamics of TPC during germination process of wheat, rye, triticale, hull-less barley and hull-less oat. Note: the values marked with different letters for each cereal type represent significant differences between values ($P < 0.05$).

Our study shows that the content of TPC increased significantly for all types of cultivars after 24 hours, except hull-less oats. It seems that oats need germinating at least for 36 hours or even more to enhance TPC content significantly. Tian et al. (2010) reported more than a 4-fold increase in phenolic compound contents in the oat after germination for 120 h. Probably, 48 h germination period for hull-less oats was too short in order to release phenolic compounds, and therefore optimization of germination process is necessary to obtain maximum content of phytochemicals. All germinated grains contained increased amounts of total phenolic content with hull-less barley having significantly ($P < 0.05$) higher content compared with the non-germinated grains (Fig. 1). The smallest increase of TPC during germination among studied grains was found in wheat and rye, while the biggest increase, approximately 2.5 times, was observed in triticale. Phenolic compounds present in cereal grains would contribute to functional and nutritional properties of the grain (Tian et al., 2010), thus increasing their nutritional value. Other studies suggested that apart from increasing total phenolic content, significant improvement in Vitamin E content has been observed for various germinated cereal grains (Kim et al., 2012; Žilić et al., 2013), primarily due to the generation of a variety of bioactive components including tocopherols and tocotrienols (Moongngarm & Saetung, 2010) and/or liberation of bound Vitamin E homologues from cellular components in grains during germination processes (Ng et al., 2013).

The degree of the changes seen in chemical composition depends on various germination conditions, such as temperature, humidity, soaking and the length of germination (Rakcejeva, 2007). According to Donkor et al. (2012) this means direct comparison is difficult, and optimum conditions will need to be defined for individual cereals. At the same time with synthesis of novel compounds, the concentrations of some nutrient inhibitors may decrease.

**Antioxidant activity**

During soaking process the DPPH scavenging activity in grains increased significantly. During the following germination process dynamics of DPPH differed among cereal types (Fig. 2). Radical scavenging activity of the phenolic extracts for non-germinated grains were between $435 \pm 16$ mM TE 100 $g^{-1}$ DW (triticale) and $680 \pm 26$ mM TE 100 $g^{-1}$ DW (hull-less barley). For 24 hours germinated grains DPPH scavenging activity ranged between $581 \pm 14$ mM TE 100 $g^{-1}$ DW (wheat) and $1232 \pm 17$ mM TE 100 $g^{-1}$ DW (hull-less barley). Even though all the grains showed substantial DPPH radical scavenging activity, hull-less barley in the germinated form appeared to have exhibited the highest activity.

During germination hydrolytic enzymes modify endosperm of components with antiradical activity (Sharma & Gujral, 2010). Enzymatic release of bound phenolics increases the TPC values during malting of barley as well as their antioxidant properties (Dvorakova et al., 2008). Cevallos-Casals & Cisneros-Zevallos (2010) repoted that significant increase of DPPH activity was determined after soaking process, whereas content of phenolic compounds in steeped seeds was lower than in raw seeds. The current research demonstrated an increase in wheat DPPH activity, with the highest activity after 12 h of germination. Hung et al. (2011) also reported that during germination wheat exhibited an increase in their antioxidant activities. The highest DPPH radical scavenging activity was detected in hull-less barley, which was in line with the study of Žilić et al. (2011) who detected higher DPPH radical scavenging ability in hull-less

barley, followed by rye and hull-less oat and durum and bread wheat, indicating that small grain species have different major antioxidants with different properties. According to Alvarez-Jubete et al. (2010) germination increase antioxidant capacity, however this may depend on cereal type, crop variety as well as of germination conditions (Gallegos-Infante et al., 2010).

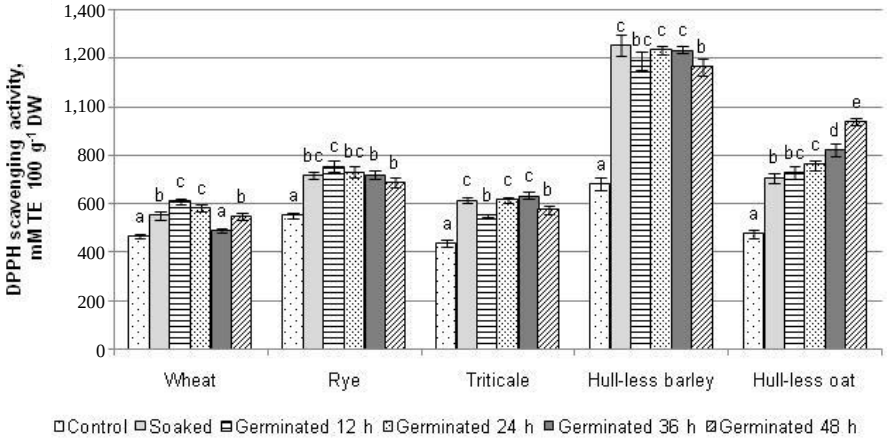


**Figure 2.** Dynamics of DPPH radical scavenging activity during germination process. Note: the values marked with different letters for each cereal type represent significant differences between values ($P < 0.05$).

ABTS radical scavenging activity after soaking process also increased significantly (Fig. 3). After 12 h of germination significant decreases were observed, except rye where decrease was measured after 24 h. In general hull-less barley grains had the highest ABTS radical scavenging activity.

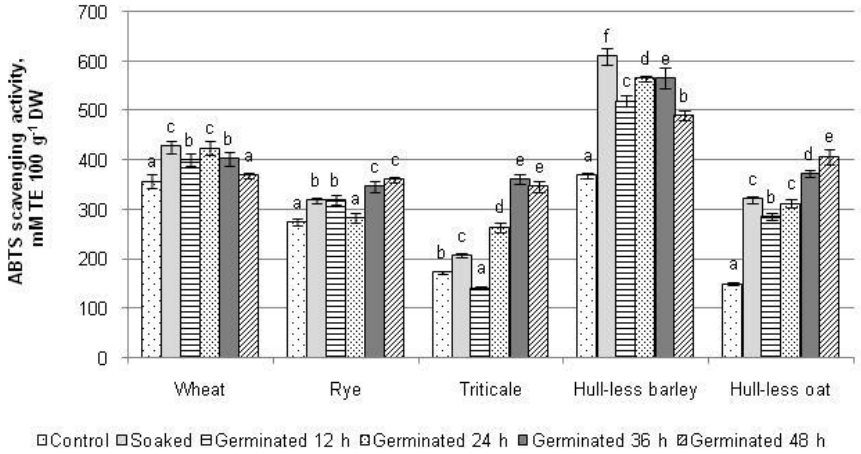


**Figure 3.** Dynamics of ABTS cation scavenging activity during germination process. Note: the values marked with different letters for each cereal type represent significant differences between values ($P < 0.05$).

**Relationships between phenolic compounds and antioxidant capacity**

Wheat, rye and hull-less barley grains containing higher levels of phenolic compounds also displayed higher scavenging activity. Similar results were reported previously (Donkor et al., 2012). However, hull-less oats had relatively low TPC among studied cereals (Fig. 1), but oat grain DPPH radical scavenging activity was comparable with activities of rye and triticale (Fig. 3). Thus radical scavenging activity in hull-less oats may be provided by phenols in combination with other compounds. Masisi et al. (2016) indicated that wide range of phytochemicals has been recognized to support overall health through their antioxidant potential. Whole grain cereals are good sources of phenolic compounds which include derivatives of benzoic and cinnamic acids, anthocyanidins, quinines, flavonols, chalcones, flavones, flavanones, and amino phenolic compounds, grains contain tocotrienols and tocopherols, and oryzanols which have antioxidant properties.

Pearson's coefficients between the phenolic compounds levels and antioxidant activity separately in each cereal type and also total correlation taking into account all obtained results are presented in Table 1.

**Table 1.** Correlation between TPC, DPPH radical scavenging activity, ABTS cation scavenging activity in grains

| Cereal | Pearson's correlation coefficient | | |
|---|---|---|---|
| | TPC/DPPH | TPC/ABTS | DPPH/ABTS |
| Wheat | 0.59* | 0.89** | 0.55 |
| Rye | 0.31 | 0.55 | 0.45 |
| Triticale | 0.82* | 0.89** | 0.56* |
| Hull-less barley | 0.48 | 0.56 | 0.94** |
| Hull-less oats | 0.65* | 0.56 | 0.98** |
| All cereals | 0.63** | 0.79** | 0.77** |

TPC/DPPH – correlation between total phenolic content and DPPH radical scavenging activity;
TPC/ABTS – correlation between total phenolic content and ABTS radical scavenging activity;
DPPH/ABTS – correlation between DPPH radical scavenging activity and ABTS radical scavenging activity
* correlation is significant at $p < 0.05$
** correlation is significant at $p < 0.01$

On average for all grain cultivars the Pearson's coefficients between the phenolic compound levels and antioxidant activity were high (TPC with DPPH and ABTS respectively $r = 0.63$ and $r = 0.79$). We found very strong positive correlations ($r = 0.82$ and $r = 0.89$) between both the content of phenolic compounds and antioxidant activity in triticale. Similar results were obtained by Bleidere et al. (2013) showing significant ($P < 0.05$) positive correlation between radical scavenging activity and total phenolic content ($r = 0.519$) in hulled barley. And also Zhao et al. (2008) reported that total phenolic content showed strong correlation with DPPH radical scavenging activity in spring barley. Opposite results were obtained by Dordevic et al. (2010) who did not find correlation between TPC and DPPH scavenging activity in the grains. Very strong correlation between TPC and ABTS scavenging activity ($r = 0.971$) was reported for commercial canola meal (Hassas-Roudsari et al., 2009) and durum ($r = 0.950$) (Žilić et al., 2012). Whereas Italian researchers analysing whole grain durum wheat (*T. durum* Desf.) determined strong correlation ($r = 0.663$) (Laus et al., 2012). Also antioxidant activity determined by both tests in our study correlated with the highest results for hull-

less barley ($r$ = 0.94) and hull-less oats ($r$ = 0.98) which allows to predict that an increase in one indicator results in increase in other indicators.

## CONCLUSIONS

The germinated grains contained significantly more phenols than non- germinated grains. The highest amounts of phenols were measured in hull-less barley grains and it was significantly higher ($P < 0.05$) than in the grains of wheat, rye, triticale and hull-less oats. However, in triticale and hull-less oats increase in phenol compounds was the highest.In order to obtain the highest TPC and antioxidant activity the optimum germination time was 24 h. The closest correlation between TPC, DPPH radical scavenging activity, ABTS cation scavenging activity was recognised in hull-less barley grains.

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# Effect of sowing date on oil, protein and glucosinolate concentration of winter oilseed rape (*Brassica napus* L.)

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**Abstract.** The effect of time of sowing on oil and meal quality of winter oilseed rape (*Brassica napus* L. cv. Express) was investigated at the Institute of Agricultural and Environmental Sciences of the Estonian University of Life Sciences in the period of 2001–2005. The rape seeds were sown at weekly intervals on four different dates: 8th, 15th, 22nd and 29th of August. The study shows that sowing date and environmental conditions affect the seed quality of winter oilseed rape. Early sown oilseed rape plants were more adapted to stressful conditions associated with high or low temperatures. The seeds of such plants had higher oil concentration (up to 50.2%) and a lower protein concentration (approximately 19%). Plants sown in late August were less tolerant to stressful conditions and their seed oil concentration was lower (47–48% DM). Oil and protein yield were higher in the early sown crops because the seed yield was higher. Also the glucosinolate (GSL) concentration of the seeds was affected by the time of sowing and weather conditions. Shortage of rainfall before harvest increased the GSL concentration in the seeds. Plants sown in late August did not tolerate the extreme environmental conditions and their seed glucosinolate concentration appeared to increase.

**Key words:** cv. Express; glucosinolate concentration; oil concentration; oil yield; protein concentration; protein yield.

## INTRODUCTION

Rapeseed cultivation has been strategically important agricultural sector for over 15 years in Estonia. It acts as profitable break crop in cereal crop rotations breaking the life-cycle of common cereal pathogens and pests and also improving the structural properties of the soil. The average yields (2.51 t $ha^{-1}$ in 2015; Statistics Estonia, 2015) have remained low compared to other European countries (EU average 3.12 t $ha^{-1}$ in 2013) (FAO, 2013), due to the large proportion (1/2) of spring rapeseed in the crop rotation. The average yield could be improved by using more winter rapeseed varieties that offer higher seed yield (3.1 t $ha^{-1}$ in 2015) (Statistics Estonia, 2015). Winter rapeseed that is usually sown at the end of summer or early autumn is characterized by more efficient use of resources (radiation, soil moisture etc) in autumn and at the start of the vegetation in spring, enabling approximately 50% yield increase compared to spring-sown rapeseed cultivars. Due to the short vegetation period in Estonia compared to Central European countries, the use of resources in early spring is of vital importance from the yield and environmental aspects (by decreasing erosion and nutrient leaching). The importance of efficient use of resources (for example, nutrients, water, temperature)

will gain more attention in the near future in the light of climate change, rising population and increasing input costs of crop production. The increased cultivation of winter rapeseed offers improved soil cover in the winter reducing the wind and water erosion and nutrient leaching in the soil (Sieling & Kage, 2010).

Rapeseed is grown worldwide in different climatic conditions. Even extreme conditions for crop growth are tolerated (Diepenbrock & Grosse, 1995). Sowing date of winter oilseed rape is an important determinant of length of growing season, insect infestation, seed and oil yields (Keshta & Leilah, 2003). Previous results Mus´nicki et al. (1999), Butkutė et al. (2006), Lääniste et al. (2007; 2008) indicate a strong relationship between growth and development of winter oilseed rape and environmental factors. Thus the sowing date has to be chosen such that yields are optimal taking negative environmental effects into account (Dejoux et al., 2003) and ensuring that leaf area and taproot reserves after the winter are sufficient to enable the crop to resume its growth quickly in spring (Mendham et al., 1981).

The major objectives of growing oilseed rape are its seed yield and seed oil concentration (Rathke & Schuster, 2001). Site-specific environmental conditions often account for large variations in seed yield and quality (oil, protein, glucosinolates) (Rathke et al., 2005). Glucosinolates (GSLs), which form a constituent of extracted meal, have antinutritive properties (Mika et al., 2003). On the other hand, some GSLs play an important role in the crop's resistance to pests and diseases (Zukalova & Vašak, 2002).

European growing technologies of winter oilseed rape vary little, with the exception of the time of the sowing, which ranges from 1st of August until 10th of September (Velička et al., 2000, Lääniste et al., 2007). According to various authors, very early sowing enhances crop growth and plants become more susceptible to frost, thus causing instability in seed yield (Graf & Heydrich, 2000, Lääniste et al., 2007, Lääniste et al., 2008). However, others have reported that plants grown from delayed sowing dates did not survive the winter (Lääniste et al., 2008) or that the seed yield was reduced (Graf & Heydrich, 2000). Compact plants with short internodes, that are in the six true-leaf stage usually show the highest survival rate (Behrens, 2002, Lääniste et al., 2007). However, under changing climatic conditions in Eastern Europe the rape sown during the second half of August (20.08) and at the end of August (30.08), whose autumnal growth and cold acclimation period was 64–76 days, was best prepared for wintering (Velička et al., 2010).

Oil and protein concentration of the seed are negatively correlated (Walton et al., 1999; Velička et al., 2011). Estonia is located on the eastern coast of the Baltic Sea. It represents a transition zone from the maritime climate type to the continental one. In spite of its comparatively small territory, climatic differences are significant, especially during the colder part of the year (Timothy & Granscog, 2001).

Field experiments were carried out to determine the effect of sowing date on seed oil, protein and glucosinolate concentration of the seeds and also oil and protein yield.

## MATERIALS AND METHODS

The crops were sown in the growing seasons of 2001–2002, 2003–2004 and 2004–2005 at the Eerika Experimental Station of Plant Biology (58°23'N, 26°44'E) near Tartu, Estonia. In each of the 3 seasons the rape seeds were sown weekly on four different dates: 8th, 15th, 22nd and 29th of August.

The soil of the experimental field is a Stagnic Luvisol (World Reference Base for Soil Resources; Deckers et al., 2002), with a texture of sandy loam and a humus layer of 20–30 cm (Reintam & Köster, 2006). Once a year before the sowing (early August), the soil samples were taken from depth of 0–25 cm The results are presented as averages of three years. Soil analyses were carried out at the laboratories of the Department of Soil Science and Agrochemistry, EMU. Air-dried soil samples were passed through a 2 mm sieve. The following characteristics were determined: pH (in 1 *M* KCl and in 0.01 *M* $CaCl_2$ 1: 2.5 w: v); mobile P and mobile K were determined by AL-method (Egner et al., 1960); Ca was determined by Mehlich III method (Mehlich, 1984) and water-soluble S was determined by ISO 11048. The soil data of the topsoil of the experimental field were as follows: pH ≈ 6.2; humus, 2.4%; Ca, 5648 mg $kg^{-1}$; P, 77.7 mg $kg^{-1}$ (Al-method; Egner et al., 1960); K, 169.8 mg $kg^{-1}$; S 13.5 mg $kg^{-1}$.

The experiments were carried out with *Brassica napus* cv. Express, bred in Germany. 'Express' is a productive cultivar with rapid autumn and spring development. The cultivar is resistant to winter conditions. Its plants have short stems, resulting in good lodging resistance.

The experiments were laid out in three replicates. Plot size was 1 m × 10 m. Seeds were sown at a rate of 150 seeds per $m^2$, at a depth of 2–3 cm, previous crop being bare fallow. Prior to sowing, the herbicide Trifluralin (EK Trifluralin, AgroDan A/S) was applied at a rate of 0.72 kg a.i. in 400 l water per ha. The field was fertilised with complex granular combined fertiliser: Classic Brand 24-08-12, calculated at 120 kg of the active substance agent of nitrogen per hectare. The fertiliser was applied in April, when the plants had reached intensive growth phase of their vegetative mass. For insect pest control, the plants were sprayed with Fastac, with alpha-cypermethrine as the active substance agent. The rate of active substance agent was calculated at 0.15 l $ha^{-1}$. The plants from test plots were harvested with a combine harvester and cleaned by a winnower. Thereafter the seeds were dried to a moisture concentration of 7% and then the yield was calculated.

The quality parameters of the seeds (oil concentration, protein concentration and glucosinolate concentration) were analyzed at Jõgeva Plant Breeding Institute laboratory using NIR (near infrared spectroscopy) technology. The oil, protein and GLS concentration were presented as percentages of dry matter.

Weather data were obtained from the automatic weather station near the Institute of Agricultural and Environmental Sciences. The quality parameters of the seeds (oil concentration, protein concentration, glucosinolate concentration) were determined using near infrared spectroscopy. Below, the oil, protein and glucosinolate (GSL) concentrations are presented as percentages on a dry matter basis.

The Statistica version 11.0 (Statsoft Inc.) software package was used for all statistical analyses. Factorial analysis of variance (ANOVA) and one-way ANOVA were applied to test the results. The means are presented with their confidence limits. The level of statistical significance was set at $P < 0.05$, if not indicated otherwise. Pearson correlation analysis was used to study correlation between seed yield and oil seed rape seeds quality parameters (glucosinolate concentration, oil concentration, protein concentration). Linear correlation coefficients between variables were calculated, the significance of coefficients being $P < 0.001$, $P < 0.01$, $P < 0.05$, ns: not significant ($P > 0.05$).

## RESULTS AND DISCUSSION

The results from our experiments indicated that the quality of winter oilseed rape seed (oil, protein, and GSL concentration) directly depends on sowing date and weather conditions during the growth period.

The weather during the experiment period was monitored with Metos Compact (Pessl Instruments) electronic weather station, which automatically calculates the average daily temperatures and the sum of precipitation. To obtain the decade average of daily average temperatures at the weather station, the daily temperatures were averaged over each decade. The weather during the period April–May of the growing seasons of 2001–2002, 2003–2004 and 2004–2005 was typical for the Estonian climate (Table 1). The season of 2001–2002 was characterised by a long active growth period in autumn (until 19$^{th}$ of October) and by early spring growth (10$^{th}$ of April). The beginning of the active growth period in spring was warm and lacked rainfall (Table 1). The season of 2003–2004 was typical for the region. The period of active growth period ended on the 14th of October and started on the 5th of April. This period was sufficiently cold. Relatively more precipitation occurred in June and July and relatively less in April and May. The second part of the growing season was extremely rainy, especially June (Table 1). The growth period in autumn of 2004 was relatively short and ended on the 10$^{th}$ of October and the active growth period in spring started relatively late (26$^{th}$ of April). The beginning of the active growth period in spring of 2005 of oilseed rape was wet but June and July were sufficiently dry (Table 1).

**Table 1.** Average monthly temperatures (°C) and precipitation (mm) during the growing period in the Eerika experimental field

| Month | Temperature, °C | | | | Precipitation, mm | | | |
|---|---|---|---|---|---|---|---|---|
| | 2002 | 2004 | 2005 | Average of 1966–1998 | 2002 | 2004 | 2005 | Average of 1966–1998 |
| April | 6.4 | 5.7 | 5.0 | 4.4 | 20.1 | 12.8 | 21.8 | 32.4 |
| May | 19.3 | 12.1 | 10.9 | 11.0 | 15.4 | 34.4 | 114.0 | 55.0 |
| June | 16.5 | 13.4 | 14.5 | 15.1 | 50.1 | 210.6 | 54.2 | 66.0 |
| July | 20.1 | 16.4 | 19.2 | 16.7 | 44.7 | 113.2 | 21.8 | 72.0 |

### Sowing date and seed oil concentration and oil yield

Many authors have reported that sowing time affects the rapeseed yield and its major quality indicators. Higher seed yield results in higher oil yield and oil content (Kirkland & Johnson, 2000; Butkutė et al., 2006). However, some authors suggest that winter oilseed rape sowing time does not affect seed quality (Zhang & Zhang, 2012). Our research indicated that the oil concentration of the seeds was mainly affected by sowing date as well as by the weather during the growth period. Walton et al. (1999) found that temperature is an important environmental factor affecting the oil and protein concentration of winter oilseed rape. The weather data for the period April–July of the seasons 2001–2002 and 2004–2005 show that the temperatures were significantly higher and the rainfall less compared to the long-time averages (Table 1). In the season 2004–2005 the temperatures in June and July were markedly lower and precipitation was higher compared to the long-time averages (3 times more in July and 1.6 times more in June) (Table 1).

Walton (1999) found that high temperatures during ripening reduced oil concentration while increasing protein concentration in a Mediterranean-type environment. In our experiments it was observed that the higher temperatures, especially in June and July, affected the synthesis of oil positively and the oil concentration of the seeds increased significantly (more than 50% oil) (Fig. 1). The seeds produced by plants that had been grown in cool and rainy weather conditions had a lower oil concentration (less than 50%). The oil concentration of the different treatments in our experiment was very high (reaching above 50%).

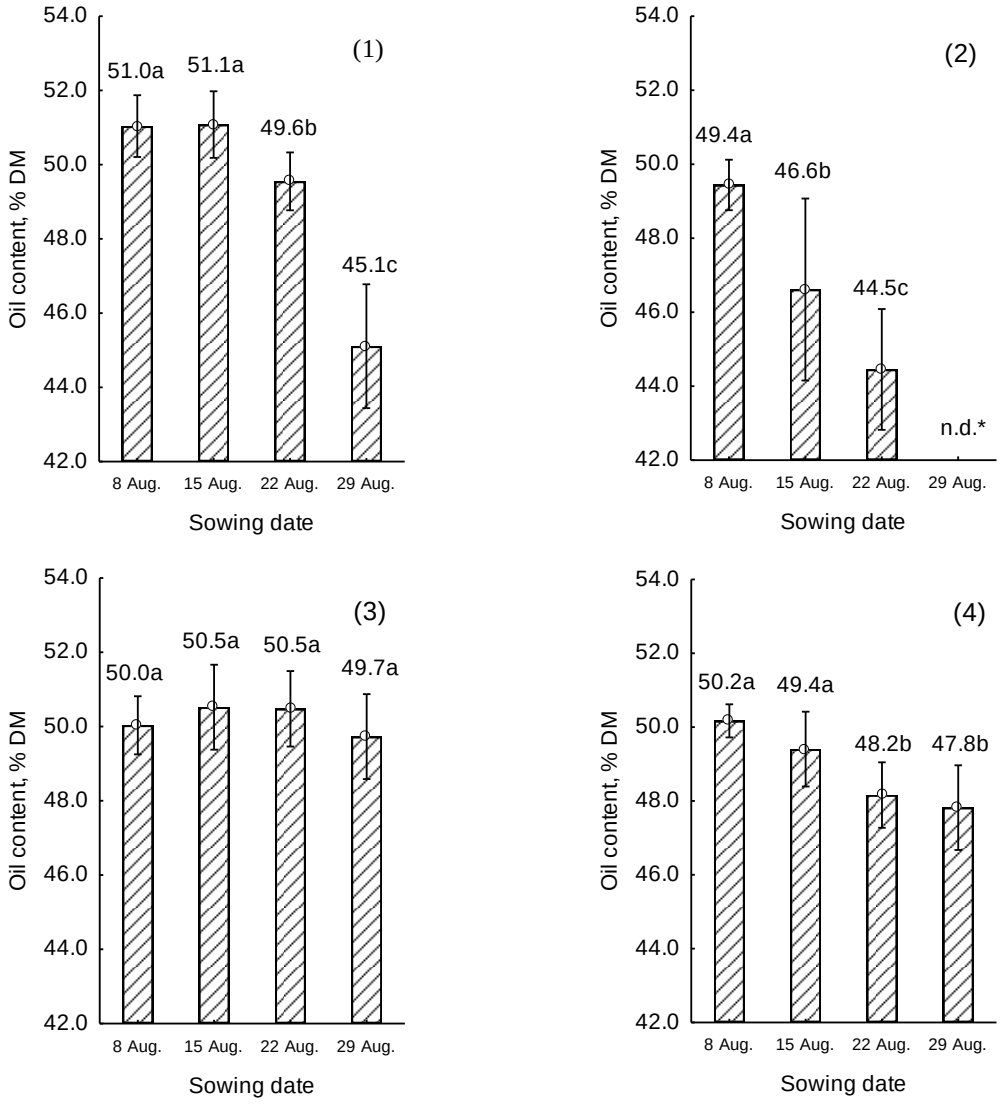


**Figure 1.** Oil concentration (% in DM) in winter oilseed rape cv. 'Express' seeds: (1) 2001–2002, (2) 2003–2004, (3) 2004–2005 and (4) average of three growing seasons. Vertical bars denote 0.95 confidence intervals. Different letters indicate significant differences ($p < 0.05$) between sowing dates. * – no seeds were harvested.

Also the oil concentration of the seeds is influenced by the time of sowing. Early sowing (8th and 15th of August) yielded seeds with an oil concentration of 50.2% and 49.4%, respectively. These data were significantly higher than the values for 22nd and 29th of August (48.2%, $p < 0.05$ and 47.8%, $p < 0.05$), respectively) (Fig. 1). The oil concentration was particularly negatively affected when the rape seeds was sown on the 29th of August and the plants were in the 3–4 leaves stage when winter started (7–8 foliage leaves is considered optimal) and also the root system was poorly developed (Lääniste et al., 2007). Such plants are very sensitive for the stress factors: extremely high/low temperatures, frost lift, excessive rainfall, and drought, and also the uptake of nutrients and water from the soil is hindered due to the poorly developed root systems. Under continuous stress conditions the synthesis of oil is inhibited.

Sowing date has an effect on the yield of rapeseed (Leach et al., 1999, Walton et al., 1999; Lääniste et al., 2008) as well as oil yield (kg ha$^{-1}$). Leach et al. (1999) demonstrated that delayed sowing increased the seed yield and oil yield in Great Britain due to the maritime climate. In contrast, several authors reported that delayed sowing decreased grain yield in northern Germany (Schulz et al., 1994, Graf & Heydrich 2000, Sieling et al., 2005) and in Estonia (Lääniste et al., 2008).

The oil yield as the main important yield parameter highly correlated ($P < 0.001$) with the seed yield. Kuht et al. (2013) observed, that the oil concentration of oilseed rape seeds was in strong negative correlation with seeds yield. A significantly higher oil yield was obtained from plants sown on the 15th of August (809 kg ha$^{-1}$, $p < 0.05$), a yield that exceeded the yield of plants sown on the 29th of August by 35%, and the yield of plants sown on 22 August by 20% (Fig. 2).

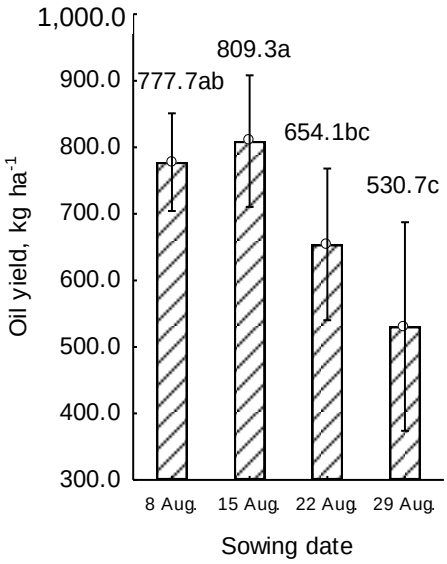


**Figure 2.** Oil yield (kg ha$^{-1}$) average of three growing seasons in winter oilseed rape cv. 'Express'. Vertical bars denote 0.95 confidence intervals. Different letters indicate significant differences ($p < 0.05$) between sowing dates.

**Sowing date and seed protein concentration and protein yield**

There is a strong negative correlation between the protein concentration and the oil concentration of the seeds (Brennan et al., 2000; Kuht et al, 2015). The protein concentration of the seeds is strongly affected by the date of sowing. The protein concentration of the seeds from plants sown late in autumn (22nd and 29th of August) was significantly higher (20.5%, $p < 0.05$ and 22.1%, $p < 0.05$ protein, respectively), compared with the seeds from early sown crops (19.2% and 19.5% protein) (Fig. 3).

Although the seed protein concentration of late sown crops was statistically higher $p < 0.05$, the seed protein yields remained lower (262.0 and 209.8 kg ha$^{-1}$) than those of the early sown crops (300.2 and 311.6 kg ha$^{-1}$) (Fig. 4). This effect can be explained by lower overall yields of late sown crops.

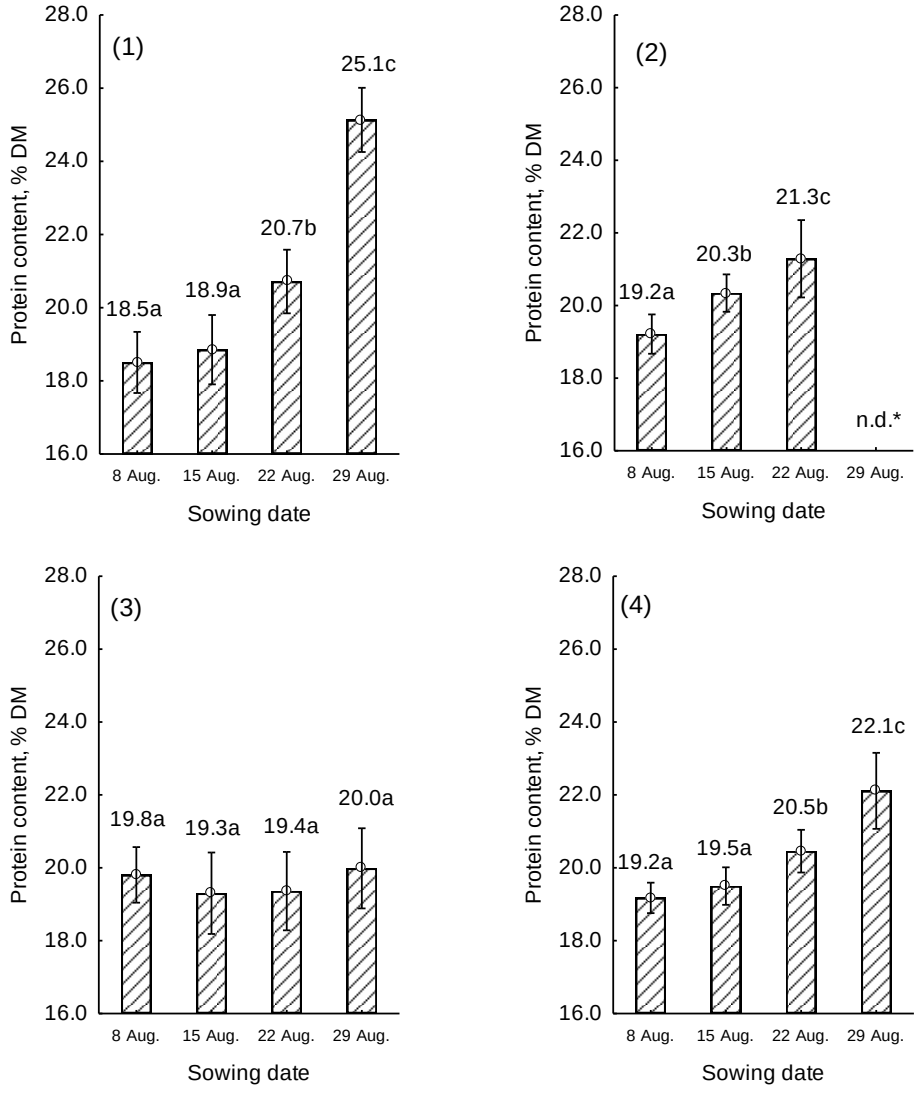


**Figure 3.** Protein concentration (% in DM) in winter oilseed rape cv. 'Express' seeds: (1) 2001–2002, (2) 2003–2004, (3) 2004–2005 and (4) average of three growing seasons. Vertical bars denote 0.95 confidence intervals. Different letters indicate significant differences ($p < 0.05$) between sowing dates. * – no seeds were harvested.

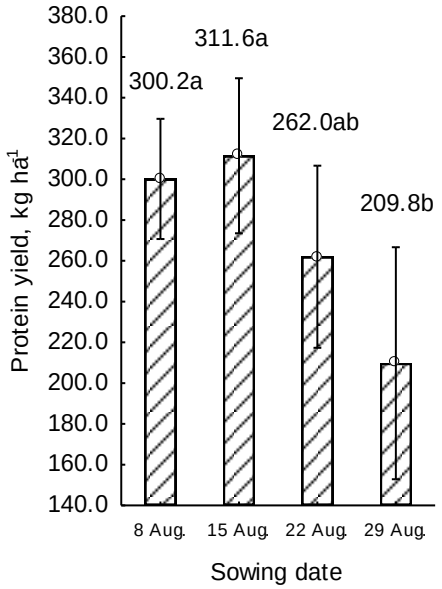


**Figure 4.** Protein yield (kg $ha^{-1}$) average of three growing seasons in winter oilseed rape cv. 'Express' seeds. Vertical bars denote 0.95 confidence intervals. Different letters indicate significant differences ($p < 0.05$) between sowing dates.

**Sowing date and seed glucosinolate concentration**

Glucosinolates are the main antinutritive components of oilseed rape seeds (Krzymanski, 1970). Breakdown products of glucosinolates, which occur during the crushing process, are partially volatile and are accumulated in circulating extraction solvents. They are chemically very active and can therefore reduce the quality and hence the value of the oil. The glucosinolate level of modern cultivars of oilseed rape is low enough to make rapeseed meal suitable for animal production (Krzymanski, 1993). The level of glucosinolate concentration depends mainly on the site, cultivar and sowing date. Generally, under optimum growth conditions nitrogen increases the glucosinolate concentration of the seeds (Bilsborrow et al., 1993).

In our experiments, the level of GSL remained low during the seasons 2001–2002 and 2004–2005 but exceeded 20 mmol per kg (Fig. 5) in 2004–2005. This suggests that drought (3.3 times less precipitation in July) before harvest tends to increase the GSL concentration of the seeds.

Date of sowing also affects the GSL concentration of the seeds. In the late sown crops (22nd and 29th of August) it increased significantly (16.4, $p < 0.05$ and 20.3 mmol per kg, $p < 0.05$ respectively) and in the early sown crops it was significantly reduced (13.8 mmol per kg) (Fig. 5). The crops sown later in autumn do not tolerate extreme environmental conditions (very cold temperatures, drought) and their glucosinolate concentration appears to increase.

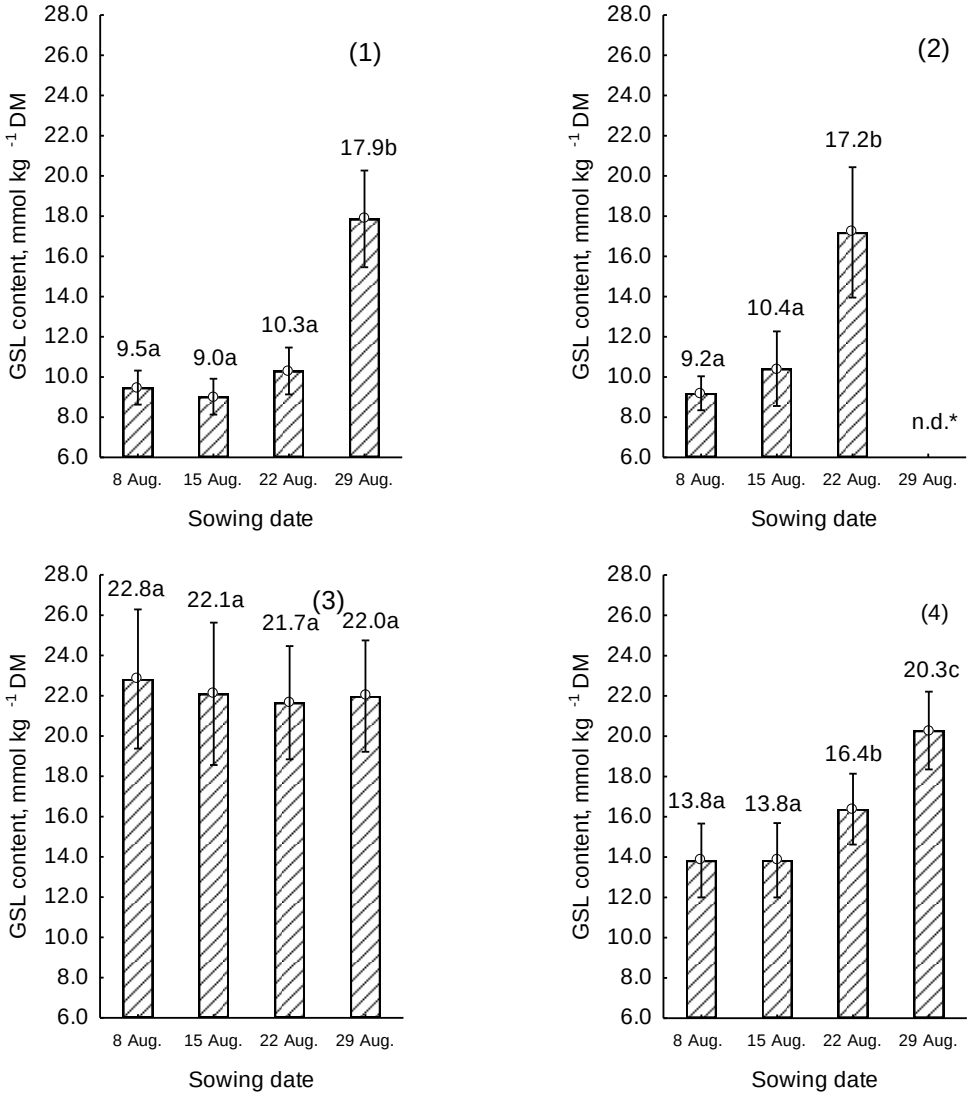


**Figure 5.** Glucosinolate concentration (mmol $kg^{-1}$ in DM) in winter oilseed rape cv. 'Express' seeds: (1) 2001–2002, (2) 2003–2004, (3) 2004–2005 and (4) average of three growing seasons. Vertical bars denote 0.95 confidence intervals. Different letters indicate significant differences ($p < 0.05$) between sowing dates. * – no seeds were harvested.

## CONCLUSIONS

This study shows that sowing date and environmental conditions affect the seed quality of winter oilseed rape. Early sowing (8th and 15th of August) yielded seeds with an oil concentration of 50.2% and 49.4%, respectively. These data were significantly higher than the values for 22nd and 29th of August (48.2% and 47.8%), respectively. The oil concentration was particularly negatively affected when the rape seeds were sown on the 29th of August and the plants were in the 3–4 leaf stage when winter started. The oil

and protein yield were higher in the early sown crops because the seed yield was higher. Also the GSL concentration of the seeds was affected by the time of sowing and weather conditions. Shortage of rainfall before harvest increased the GSL concentration in the seeds. Plants sown in late August did not tolerate the extreme environmental conditions and their seed glucosinolate concentration appeared to increase.

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# The hygienic and nutritional quality of milk from Saanen goats bred in the Moravian-Silesian region

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**Abstract.** The aim of the study was to monitor milk yield and the hygienic and nutritional quality of milk of Saanen goats in the Moravian-Silesian region in Czech Republic. Milk samples were collected once a month during the lactation period. The average milk yield in the standardized lactation was 1,100 liters. The somatic cell count in pool samples ranged from 470 x $10^3$ to 696 x $10^3$. The total microorganism count ranged from 3.6 x $10^3$ to 1.4 x $10^5$. The pathogen *Staphylococcus aureus* was proven no more than in 6.3%. The highest values of all main components of milk were achieved within a relatively short time after kidding (April 2015). The average content of fat was 3.64 ± 0.52 g 100 $ml^{-1}$, 3.17 ± 0.16 g 100 $ml^{-1}$ of protein, 2.60 ± 0.06 g 100 $ml^{-1}$ of casein, 4.56 ± 0.24 g 100 $ml^{-1}$ of lactose, and 12.02 ± 0.80 g 100 $ml^{-1}$ of solids. Average content of vitamin A was 0.27 ± 0.14 mg $kg^{-1}$ and average content of vitamin E was 0.60 ± 0.34 mg $kg^{-1}$. Content of vitamin E increased almost continuously during the lactation, and the content of vitamin A was significantly higher at the end of lactation. In lyophilized milk powder the average trace metal contents were 7.76 ± 0.92 g $kg^{-1}$ Ca, 1.62 ± 0.26 g $kg^{-1}$ Mg, 15.3 ± 1.43 g $kg^{-1}$ K, 789 ± 111 mg $kg^{-1}$ Na, 23.2 ± 2.73 mg $kg^{-1}$ Zn, and 0.85 ± 0.55 mg $kg^{-1}$ Cu. Contents of minerals varied during the lactation period, but no significant trends were observed.

**Keywords:** total microorganism count, somatic cell count, pathogens, fat, protein, casein, lactose, vitamin A, vitamin E, Ca, Cu, K, Mg, Na, Zn.

## INTRODUCTION

Goat breeding has recently expanded in the Czech Republic, primarily on private farms with direct dairy production. Consumers consider goats to be ecologically bred animals, and their specific taste is increasingly adapted to maintain human health. A similar trend can also be observed in neighbouring Austria (Mayer, 2005). Goat milk is recommended as a substitute for cow's milk, especially for people suffering from allergies to cow's milk. In human nutrition, goat milk has hypoallergenic and therapeutic effects (Park et al., 2007). The size of the fat globules is smaller in goat milk than in cow's milk, meaning it is more easily digested (Iannotti et al., 2013).

The composition of macro- and micro-nutrients in goat milk depends on the main production factors constituting the farming system, such as genotype, reproduction and sanitary characteristics of animals, agro-climatic conditions and the socio-economical

environment, and the farming methods used during feeding and milking. Actually, the link between these factors can be close and complex (Addis et al., 2005, Morand-Fehr, 2005). The content of macro- and micro-nutrients depends also on the lactation stage (Hejtmánková et al., 2012, Michlová et al., 2015) and on the region of production (Kedzierska-Matysek et al., 2013).

Goat milk and goat dairy products are relatively new still more common products in human nutrition in the Czech Republic, but legislative requirements on the quality of goat milk do not yet exist. Additional information is therefore required regarding the hygienic quality and nutritional composition of raw milk at least.

The farming of Saanen goats was established in the Czech Republic in 2014. This is the best breed used for the production of dairy goats. Annual production of milk per lactation period corresponds to 20 times the animal body weight, and ranges from 300–2,000 kg in 150–300 days of lactation, depending on country. In leading countries the average is above 975 kg (Roginski et al., 2003). An increasing number of people are showing interest in goat milk, and due to the high milk yield of this breed, it seems the breeding of Saanen goats is economically appealing.

The aim of this study was to monitor the milk yield and the hygienic and nutritional quality of milk from Saanen goats in Moravian-Silesian region of the Czech Republic.

## MATERIALS AND METHODS

### Experimental material

The basic composition (protein, fat, lactose, solids, non-fat milk solids), content of casein, urea, vitamin E and A, selected minerals (Ca, Cu, K, Mg, K, Na, and Zn), and total microorganisms and somatic cells were determined in pool samples of Saanen goat milk from one herd that was bred in northern Moravia (Czech Republic). Monitored breeding, including 265 goats brought from the French region of Vende, is conventional, with a stable feeding ration (corn silage, hay, haylage, sugar beet, protein concentrate) and addition of probiotics that contain microorganisms such as *Saccharomyces cerevisiae* during the entire year. The mixture is stirred in feeding car Faresin. The granulated mineral licks (Caprin mix in the batch 4 g per day, Inframix s. r. o., CR) with higher concentrations of iodine are also added. In addition to the aforementioned ration, the goats received sodium bicarbonate at a dose of 50 g per head and day, as well as calcium bicarbonate and sodium chloride (*ad libitum*). All breeding females were in the first lactation. The goats were milked twice a day at regular intervals, using a BouMatic (BouMatic, USA) milking machine.

Pooled milk samples were collected once a month during the lactation period from April to September 2015. Random individual milk samples were collected monthly from May to September (approx. 15% of milked goats) for the purpose of detecting mastitis pathogens. Additionally, the ability of the milk to ferment was tested 3 times during the lactation period (July, August, and September).

Samples were collected into clean plastic 100ml sampling flasks, cooled to 4–6 °C and transferred in a thermobox to the laboratory. To ensure the homogeneity of the sample, the sampling flasks were thoroughly shaken for 2 min prior to measurement. All samples were analyzed in 3 parallel replications.

**The chemicals**

For the preparation of analytical samples, the following special standards and chemicals were used: DL-α-tocopherol, 98.2% (CALBIOCHEM, Canada), tocopherol set (CALBIOCHEM, Canada), retinol, > 99% (Sigma-Aldrich, Germany), pyrocatechol, > 99.5% (Sigma-Aldrich, Germany), methanol, super gradient, content min. 99.9% (Lachner, Czech Republic). Standard solutions ASTASOL (Analytika, CR) of Ca, Cu, K, Mg, Na, and Zn (1 g $l^{-1}$) prepared in treated distilled water (Millipore, France) were used in the preparation of calibration curves for the measurements. All commonly used chemical were of p.a. quality.

**The methods**

**Measurement of basic components of milk including casein and urea** was performed using a DairySpec FT analyzer (Bentley Instruments, Inc., USA).

Measurement of somatic cell count was performed using a DeLaval Cell counter (DCC De Laval International AB, Sweden).

Detection of inhibitors in milk was carried out using a microbiological broadspectrum inhibitor test by Delvotest T (Reybroeck & Ooghe, 2012, Sats et al., 2014).

Determination of pathogenic microorganisms was carried out by VEDIA s.r.o., (Strakonice, CR – accredited laboratory of the State Veterinary Administration of the Czech Republic; established according to the Veterinary Act No. 166/1999).

**Measurement of vitamins A and E content in milk samples.** Both vitamins were extracted using the method of Sánchez-Machado et al. (2006) with minor modifications by Michlová et al. (2015). The analysis was carried out using an Ultimate 3000 High Performance Liquid Chromatograph (Thermo Fisher Scientific, Dionex, Sunnyvale, USA) with a quaternary pump, refrigerated autosampler, column heater and FLD and DAD detectors. Tocols and retinol in the sample were determined by HPLC under the following conditions: analytical column Develosil 5 μm RP AQUEOUS (250×4.6 mm) (Phenomenex, Torrance, USA); isocratic elution, mobile phase methanol: deionised water (93:3, v/v) (Michlová et al. (2015). All results were expressed as mean values of three replicates.

**Measurement of minerals.** For the determination of Ca, Cu, Mg, K, Na, and Zn in milk, aliquots of frozen milk samples (50 ml) were lyophilized using a LYOVAC GT 2 (LEYBOLD-HERAEUS, GmbH, Germany) and then approx. 0.8 g of lyophilized milk was mineralized by dry ashing (Mader et al., 1997; 1998). Analyses were carried out in triplicate.

Concentrations of Ca, K, Mg, Na, and Zn in the digests were determined by flame atomic absorption spectrometry (FAAS) using a Varian SpectrAA 110 instrument (Varian, Inc., Mulgrave, Victoria, Agilent Technologies Inc., Palo Alto, CA, USA) in an acetylene-air flame at wavelengths 766.5 nm (K), 589.0 nm (Na), 422.7 nm (Ca), 285.2 nm (Mg) and 213.9 nm (Zn). The widths of spectral intervals were 1 nm (K, Na and Zn) and 0.5 nm (Ca and Mg).

During the measurement of Mg and Zn the background was corrected by a deuterium lamp. In the determination of Ca and Mg, 1% solution of lanthanum nitrate was added as a releasing agent. SIPS (Sample Introduction Pump System) was used for the creation of calibration dependence.

Concentrations of Cu in the digests were measured by electrothermal atomic absorption spectrometry (ETAAS) using a Varian AA 280Z (Varian, Belrose, Australia) with graphite tube atomizer GTA 120 and PSD 120 programmable sample dispenser at the wavelength 324.8 nm. Detailed temperature programs for the determination of Cu in milk are described in the Technical Report (Mader et al., 2000). The quality of analytical data was assessed by simultaneous analysis of certified reference material CRM 063R (Skim milk powder) (3.3% of all the samples). Analytical data obtained for all determined elements were found to be within the confidence interval given by the producer of the CRM.

**The fermentation ability was tested using a yoghurt** culture (WV2) based on the bacterial strain CCDM 176 (Milcom a.s. Laktoflora, CR). Milk samples used to cultivate the yogurt culture were first heat-treated at 85°C for 10 min. After heat treatment, the samples were colled down to a temperature of 30 °C and inoculated by 0.1% yoghurt culture CCDM 176 to 100 ml of milk. Cultivation was carried out at 30°C for 16–19 hours. Culture mediums RS 5, 4, and M17 (Milcom a.s., Laktoflora, CR) at pH = 5.54 were used for the determination of yogurt bacterium.

The determination of colony forming units in yogurts was executed acording to the International Standard ISO 7889:2003 (Yogurt - Enumeration of characteristic microorganisms - Colony-count technique at 37 °C.) In addition, the active acidity was measured using a pH meter (Schott, SI Analytics, Germany).

**Statistical analysis** was performed using Statistica Version 9 (2009). The measured values were processed by analysis of variance (ANOVA), using the post-hoc Tukey's test.

## RESULTS AND DISCUSSION

The average milk yield in the standardized lactation from the monitored herd of Saanen goats was 1,100 liters. Torres-Vázquez et al. (2009) observed almost the same milk yield (1,095 ± 292 liters at the first lactation) in Saanen goats from Mexico. These values are slightly higher than the average value of 975 kg given by Roginski et al. (2003), and it seems to be convenient amount in the conditions of the Czech Republic. Indicative preliminary results confirmed the ability of milk to ferment. The microorganism count of yogurt culture in the final product ranged from $9.0 \times 10^7$ to $3.7 \times 10^8$. The pH shifted from 3.9 to 4.2.

### Hygienic quality of milk

The basic qualitative assessment of goat milk is based on the parameters of the Slovak technical standard STN 57 0520 GOAT MILK (1995), and Council Directive 92/46/EEC and of annex No.1 of the law 203/2003 Coll. on animal health requirements for milk. The Slovak standard for goat milk contains only two requirements – total microorganism count (TMC) $\leq 500 \times 10^3$ ml$^{-1}$ and number of colony forming units (CFU) of Staphylococcus aureus $\leq 2 \times 10^3$ ml$^{-1}$. According to Kautz et al. (2014) the legal somatic cells count (SCC) limit for herd milk in dairy goats is $1{,}500 \times 10^3$ ml$^{-1}$. Any similar standard does still not exist in the Czech Republic.

The qualitative assessment of pooled samples of raw milk is given in Table 1. Active acidity of milk ranged from pH 6.62 to 7.22. The mean value was 6.904 ± 0.248; this is higher than the value of 6.60 ± 0.11 given by Trancoso et al. (2010).

**Table 1.** Qualitative assessment of the raw milk – pooled samples

| Month | TMC | Psychroph. bacteria | Thermo resistant bacteria | Spore producing bacteria | Coliform bacteria | SCC |
|---|---|---|---|---|---|---|
| | (CFU $ml^{-1}$) | (CFU $ml^{-1}$) | (CFU $ml^{-1}$) | (CFU $ml^{-1}$) | (CFU $ml^{-1}$) | ($10^3$ $ml^{-1}$) |
| May | $3.5 \times 10^4$ | <10 | $4.0.10^1$ | $1.0 \times 10^1$ | <10 | 570 |
| June | $3.6 \times 10^3$ | <10 | <10 | $3.1 \times 10^2$ | <10 | 470 |
| July | $5.8 \times 10^3$ | <10 | <10 | $3.2 \times 10^3$ | <10 | 531 |
| August | $5.1 \times 10^3$ | <10 | <10 | $2.3 \times 10^3$ | <10 | 696 |
| September | $1.4 \times 10^5$ | <10 | <10 | $1.8 \times 10^4$ | <10 | 541 |

Screening of milk for the presence of inhibitors was in all cases negative. Unfortunately *Staphylococcus aureus* was also detected in June and July. In these months the highest presence of *Staphylococcus aureus* in milk was recorded (200 CFU $ml^{-1}$). This value is still 10 times lower than the requirement of the Slovak standard. In addition, Polish dairies tolerate SCC up to the value of $800 \times 10^3$ $ml^{-1}$. A value above $4{,}000 \times 10^3$ $ml^{-1}$ is penalized. Acording to these criteria, milk from the monitored farm has very high hyginic quality; SCC ranged from $470 \times 10^3$ $ml^{-1}$ to $696 \times 10^3$ $ml^{-1}$ and $510 \times 10^3$ $ml^{-1}$ on average.

The goats' milk secretion system differs from that of the cow (Hinckley, 1990), and evidence indicates several basic differences between the composition of goat milk and cow milk. In a monitored heard of Saanen goat (with exception of September), TMC in milk complies also with the requirements of cow's milk (TMC at 30 °C ≤ $100 \times 10^3$ $ml^{-1}$ and SCC ≤ $400 \times 10^3$ $ml^{-1}$ according to Regulation of the EP and of the Council (EC) No. 853/2004). SCC in the monitored Saanen goat herd was higher in all cases. According to Hinckley (1990), differences between cows and goats depend also on other requirements, especially on the somatic cell count standard in goat milk. Nevertheless, SCC in the monitord heard was relatively low. In some other studies, the determined SCCs in goat Saanen milk were higher (Laurinaviciute et al, 2004, Vilanova et al., 2008, Kautz et al., 2014).

A positive health status of the mammary glands of goats is reflected in the number of sterile samples (Table 2). Major environmental pathogens *Staphylococcus* PK-(delta hemolysin+) and *Staphylococcus* PK-(delta hemolysin-) were identified in some pool raw milk samples in each of the studied months. *Streptococcus uberis*, *Enterococcus* sp., *Aeromonas* sp., coliform bacteria, and aerobic sporulate were also determined once during the monitoring period. The ongoing lactation and isolation of the goats in the stable, and the applied system of hygiene reduced the number of sterile samples and environmental germs by about 50%, compared to the input values. The opposite trend was apparent for contagious embryos, whose main representative is *Staphylococcus aureus*. However, it is necessary to say that *Staphylococcus aureus* was diagnosed in the mammary gland of goats and the characterization of its exposure is definitely negative. The pathogen easily expands and can cause an infectious disease. Therefore, economic losses on milk production and its composition quality are high.

**Table 2.** Diagnostics of the presence of pathogens (in %) – individual milk samples

| Month | Sample (n) | Sterile sample | Environmental pathogens<br>*Staphylococcus* PK – (delta hemolysin +)<br>*Staphylococcus* PK – (delta hemolysin -) | *Staphylococcus aureus* |
|---|---|---|---|---|
| May | 48 | 72.9 | 20.8 | 6.3 |
| June | 48 | 79.2 | 16.7 | 4.1 |
| July | 40 | 75.0 | 22.5 | 2.5 |
| August | 40 | 50.0 | 50.0 | 0.0 |
| September | 36 | 69.4 | 27.8 | 2.8 |

It is valuable, that test results found no positive inhibitors in the current study. TMC, SCC (Table 1) and CFUs of *Staphylococcus aureus* in milk from the monitored farm correspond to the above given criteria.

Hygienic preparation of the mammary glands for milking, and disinfection treatment after milking did not exist until the determination of the presence of pathogens. In connection with the findings of mastitis pathogens in individual milk samples, the practice of disinfecting the milking machine using peracetic acid solution was introduced. Therefore, we can expect that the presence of pathogens will be reduced in future.

**Main constituents of milk**

The main components determined in Saanen goat milk are shown in Table 3. The content of main nutrients changed during the lactation period. The highest values were achieved within a relatively short time after kidding, in April. This applies to all components of the milk (i.e., fat, protein, casein and lactose), when their levels were comparable with the composition of cow's milk. Values in the summer months were marked by extreme heat, resulting in reduced content. The last collection in September, once again brought an increase in values, however, they did not reach the levels of April. The lowest observed level of urea was in April, followed by an increase in May and June, and then a slight decrease in the remaining months. In high concentrations, urea has negative effects, manifested as a reduction of immune reactions.

Hinckley (1990), Antunac et al. (2001a; 2001b), and Vilanova et al. (2008), reported lower contents of crude protein, fat, lactose and solids in Saanen goat milk. Only Trancoso et al. (2010) determined a higher content of fat ($5.01 \pm 1.1\%$), protein ($3.75 \pm 0.19\%$) and solids ($13.16 \pm 1.3\%$). According to Kozacinski et al. (2004) an increase in SCC was associated with an increase in protein and non-fat solids, and a reduction in lactose and milk fat content. However, in this study, the SCC was the highest in the middle of lactation, and in contrast, the content of protein and non-fat solids were the lowest. Changes observed in the composition of milk during the lactation period are in accordance with Antunac et al. (2001a), who found a significantly higher ($P < 0.01$) content of dry matter, non-fat solids, and lactose at the beginning of lactation in comparison with the middle of lactation. Significant correlations ($P < 0.001$) were established between the content of dry matter and the content of non-fat solids (0.76%), fat (0.77 g 100 $ml^{-1}$), protein (0.64%) and lactose (0.46%). At the end of the lactation period (day 200), the content of protein (3.11%) and solids (11.76%) were higher than at the first quarter of the lactation period (day 50) (2.81% and 11.91% respectively) (Antunac et al., 2001b). The same trend was observed in this study. At the end of the

lactation period, the content of fat, protein, casein and solids were higher than at the middle of lactation (Table 3).

**Table 3.** The main composition of Saanen goat milk

| Month | Fat (g 100ml$^{-1}$) | Protein (%) | Casein (%) | Lactose (%) | Solids (%) | Solids non-fat (%) | Urea (mg l$^{-1}$) |
|---|---|---|---|---|---|---|---|
| April | 4.50 | 3.47 | 2.71 | 5.00 | 13.46 | 9.14 | 321 |
| May | 3.81 | 3.06 | 2.55 | 4.62 | 12.18 | 8.43 | 648 |
| June | 2.91 | 3.06 | 2.54 | 4.48 | 11.14 | 8.27 | 612 |
| July | 3.52 | 3.12 | 2.58 | 4.53 | 11.86 | 8.38 | 467 |
| August | 3.41 | 3.11 | 2.61 | 4.29 | 11.48 | 8.27 | 509 |
| September | 3.70 | 3.21 | 2.63 | 4.46 | 11.98 | 8.53 | 429 |
| Average | 3.64 | 3.17 | 2.60 | 4.56 | 12.02 | 8.50 | 498 |
| SD | 0.52 | 0.16 | 0.06 | 0.24 | 0.80 | 0.33 | 121 |
| $S_r$ (%) | 14.29 | 5.05 | 2.31 | 5.26 | 6.66 | 3.88 | 24.2 |
| Maximum | 4.50 | 3.47 | 2.71 | 5.00 | 13.46 | 9.14 | 648 |
| Minimum | 2.91 | 3.06 | 2.61 | 4.29 | 11.14 | 8.27 | 321 |

**Vitamin contents**

The content of vitamin A and E in Saanen goat milk are given in Table 4. In agreement with previous determinations of vitamin A and E in the goat milk (Michlová et al., 2015), the average content of vitamin E in milk of Saanen goats was higher (2.2 times) than the content of vitamin A. However, the average contents of both vitamins were significantly lower, although the fat content in milk was comparable with the fat in the milk of other goat breeds (with the exception of Anglo Nubian goats). The average content of vitamin A was $0.27 \pm 0.14$ mg kg$^{-1}$ ($0.79 \pm 0.08$ mg kg$^{-1}$ in the milk of various breeds of goats from different farms), and the average content of vitamin E was $0.60 \pm 0.34$ mg kg$^{-1}$ ($1.29 \pm 0.35$ mg kg$^{-1}$).

**Table 4.** Content of vitamin A and E in raw Saanen goat milk

| Month | Vitamin A (mg kg$^{-1}$) | Vitamin E (mg kg$^{-1}$) |
|---|---|---|
| April | 0.18 | 0.19 |
| May | 0.21 | 0.31 |
| June | 0.20 | 0.40 |
| July | 0.18 | 0.83 |
| August | 0.36 | 0.81 |
| September | 0.52 | 1.05 |
| Average | 0.27 | 0.60 |
| SD | 0.14 | 0.34 |
| $S_r$ (%) | 51.8 | 56.70 |
| Maximum | 0.52 | 1.05 |
| Minimum | 0.18 | 0.19 |

Higher levels of both vitamins in goat milk are also given by others authors (e.g., Morand-Fehr et al., 2007, Park et al., 2007). The aforementioned authors reported very high values of both vitamins (up to 11 mg kg$^{-1}$ of vitamin E and up to 6 mg kg$^{-1}$ of vitamin A) depending on the farming and feeding system. On the contrary, a lower value of vitamin E (0.4 mg kg$^{-1}$) in goat milk was reported by Raynal-Ljutovac et al. (2008),

and lower levels of vitamin A (0.13 mg $l^{-1}$) were also observed by Kondyli et al. (2012). In this study, the content of vitamin E increased almost continuously during the lactation, and the content of vitamin A was also significantly higher at the end of the lactation period.

**Mineral contents**

Contents of Ca, Cu, K, Mg, Na, and Zn in Saanen goat milk are given in Table 5. The values are related to lyophilized milk powder. Despite the fact that the feeding ratio was stable, all measured minerals showed significant variations during the lactation period. Ca and Cu contents were significantly higher at the beginning of the lactation period. The same trend was observed by Aganga et al. (2002) and Kondyli et al. (2007). On the contrary, contents of Mg, K and Na were significantly lower at the beginning of the lactation period, but only Mg increased continuously during the entire lactation period. The average Ca content in lyophilized milk powder was 7.76 ± 0.92 mg $kg^{-1}$; lower than the value of 10.9 ± 0.8 mg $kg^{-1}$ d.w, as reported by Trancoso et al. (2010) and Antunac et al. (2001a, b) (0.110–0.129% in raw milk). Higher values of Ca content in the milk of other goat breeds are reported by many authors (Kondyli et al., 2007, Park et al. 2007, Mayer & Fiechter 2012, Kedzierska-Matysek et al. 2013, and others). The average Mg content in lyophilized milk powder was 1.62 ± 0.3 mg $kg^{-1}$, higher than that reported for Saanen goat milk by Trancoso et al. (2010) (1.03 ± 0.10 mg $kg^{-1}$ d.w.). The Mg content found in this study was consistent with that reported by Kondyli et al. (2007) and Park et al. (2007), and higher than those reported by other authors (Aganga et al., 2002, Hejtmánková et al., 2002, Mayer & Fiechter, 2012). The average Na content in lyophilized milk powder was 790 ± 111 mg $kg^{-1}$, significantly lower than that reported by Trancoso et al. (2010) for Saanen goat milk (2.83 ± 0.4 g $kg^{-1}$ d.w.). Aganga et al. (2002), Kondyli et al. (2007), Park et al. (2007), Mayer & Fiechter (2012) and Kedzierska-Matysek et al. (2013) all reported in accordance with the aforementioned higher value of Na content in goat milk. The average K content in lyophilized milk powder for Saanen goat milk was 15.27 ± 1.43 mg $kg^{-1}$, higher than reported by Trancoso et al. (2010) (12.2 ± 2.0 g $kg^{-1}$ d.w.). The K content found in this study is similar to those reported by Aganga et al. (2002), Kondyli et al. (2007) and Park et al. (2007). Aganga et al. (2002) reported the highest value of K (0.487 g 100 $g^{-1}$ in Tswana goat milk).

**Table 5.** Content of selected minerals in lyophilized Saanen goat milk powder

| Month | Ca (g $kg^{-1}$) | Cu (mg $kg^{-1}$) | K (g $kg^{-1}$) | Mg (g $kg^{-1}$) | Na (mg $kg^{-1}$) | Zn (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|
| April | 9.41 | 1.94 | 13.50 | 1.24 | 649 | 26.40 |
| May | 7.72 | 0.73 | 13.90 | 1.48 | 691 | 20.90 |
| June | 6.91 | 0.75 | 16.30 | 1.60 | 825 | 26.70 |
| July | 6.88 | 0.46 | 14.60I | 1.68 | 851 | 23.30 |
| August | 7.88 | 0.78 | 17.10 | 1.75 | 951 | 20.80 |
| September | 7.76 | 0.45 | 16.10 | 2.00 | 769 | 21.30 |
| Average | 7.76 | 0.85 | 15.30 | 1.62 | 790 | 23.20 |
| SD | 0.92 | 0.55 | 1.43 | 0.26 | 111 | 2.73 |
| $S_r$ (%) | 11.90 | 64.70 | 9.36 | 16.0 | 14.0 | 11.70 |
| Maximum | 9.41 | 1.94 | 17.1I | 2.00 | 951 | 26.70 |
| Minimum | 6.88 | 0.45 | 13.50 | 1.24 | 649 | 20.80 |

The average Na/K ratio was lower than that reported Trancoso et al. (2010) (0.05 vs 0.23), and also lower than the ratios reported by Kondyli et al. (2007), Park et al. (2007) and Raynal-Ljutovac et al. (2008). This low Na/K ratio might be of interest from the human nutrition point of view, especially for people suffering from high blood pressure or those under dialysis (Trancoso et al., 2010). The average Zn content in lyophilized milk powder from Saanen goat milk was $23.22 \pm 2.73$ mg $kg^{-1}$, which is similar to those reported by Trancoso et al. (2010) ($26.0 \pm 4.90$ mg $kg^{-1}$ d.w.) and by Haenlein & Anke (2011). Coni et al. (1996) reported a lower content of Zn in goat milk. In contrast, Elmastas et al. (2005), Kondyli et al. (2007), Kedzierska-Matysek et al. (2013) and especially Park et al. (2007), and Sanz-Ceballos et al. (2009), give higher values. The highest value (8.79 mg $kg^{-1}$) was reported by Aganga et al. (2002). The average Cu content in lyophilized milk powder was $0.85 \pm 0.55$ mg $kg^{-1}$, slightly higher than the value (663 μg $kg^{-1}$ d.w.) reported by Trancoso et al. (2010) for Saanen goat milk and by Coni et al. (1996) for goat milk. The values determined in this study are consistent with values reported by Hejtmánková et al. (2002) and Kodyli et al. (2007). On the contrary, Elmastas et al. (2005), Park et al. (2007), Sanz-Ceballos et al. (2009) and Kedzierska-Matysek et al. (2013) gave higher values. The highest value (2.0 mg·$kg^{-1}$) was reported by Aganga et al. (2002).

## CONCLUSION

Milk from Saanen goats bred in the Moravian-Silesian region in the Czech Republic can be considered to be of good hygienic quality, and relatively high content of the main nutrients, such as protein, fat, lactose and therefore the total dry matter. The high density of animals, the quality of the used milking machine, and a higher level of sanitary measures positively influenced the somatic cells and their count, and the total microorganisms count in raw milk. Important micronutrients, particularly vitamins A and E, were found to be less concentrated than would be typically expected in goat milk. Contents of Ca and Na were significantly lower, contents of Cu, K, and Zn close to the lower boundary of the values, and the content of Mg was average in comparison with the contents of these elements reported by various authors for goat milk. However, this situation may be solved in the future by making appropriate changes to the application of additives in feeding rations. On the contrary, a low Na/K ratio might be of interest from the human nutrition point of view, especially for people suffering from high blood pressure or for those under dialysis. Despite the fact that the feeding ratio was stable, measured contents of the main nutrients, vitamins and minerals were variable during the lactation period.

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# The content of minerals in milk of small ruminants

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**Abstract.** The aim of this study was to determine and compare the content of sodium, potassium, calcium, magnesium, zinc, copper, lead, and cadmium in sheep and goat milk of different breeds from 9 farms in the Czech Republic (herds of 18–330 goat´s heads and 30–380 sheep heads). Pool samples of milk were collected once a month (April - September) during lactation in the years 2011–2013. The content of minerals was determined using atomic absorption spectroscopy. Most of the analyses of the contents of Cd and Pb were below the limit of detection. Other determined values of these two contaminants were lower than maximal tolerable amount according to previously valid regulation No. 298/1997 Sb of the Ministry of Health of the Czech Republic given for cow's milk. The contents of each element in the whole of the reference period were in a relatively wide range. Determined levels of Ca, Mg, K, Na, Zn and Cu in goat milk related to the weight of lyophilized milk powder varied from 1.40–8.08 g $kg^{-1}$, 0.16–1.42 g $kg^{-1}$, 8.16–31.10 g $kg^{-1}$, 0.72–5.43 g $kg^{-1}$, 7.59–44.10 mg $kg^{-1}$, and 0.21–1.46 mg $kg^{-1}$ respectively. Determined levels of Ca, Mg, K, Na, Zn and Cu in sheep milk varied from 1.69–9.13 g $kg^{-1}$, 0.21–1.36 g $kg^{-1}$, 3.53–11.90 g $kg^{-1}$, 0.65–5.05 g $kg^{-1}$, 13.70–34.30 mg $kg^{-1}$, and 0.15–2.10 mg $kg^{-1}$ respectively. Statistically higher ($P < 0.05$) content of potassium was determined in goat milk in comparison with sheep milk. The contents of all followed minerals in milk samples from each farm collected during the lactation period were very variable, but it is not possible to find any direct relationship between the content of studied elements and the date of sampling. It was found that the year has statistically significant influence especially on the content of Ca and Mg in milk of small ruminants.

**Key words:** goat milk, sheep milk, Ca, Cd, Cu, K, Mg, Na, Pb, Zn.

## INTRODUCTION

The milk and milk products of different ruminant species comprise a food of outstanding importance for human nutrition throughout their lives. Milk can be considered a source of macro- and micronutrients including the mineral elements, and also contains a number of active compounds that play a significant role in both nutrition and health protection (Sanz Ceballos et al., 2009). Recently in the Czech Republic the production of goat and sheep milk increases as well as all over the world. Goats and sheep are mostly bred in small scale in private farms where milk is also subsequently processed (mainly to the cheese and yoghurt). Breeding of sheep and goats is by general public associated with a friendlier attitude towards animals and nature, so that milk of small ruminant is considered as organic. With the increasingly growing network of

farmers' markets is much easier to use these products also in cities. In addition, due to the better digestibility of goat milk proteins this milk plays an important role in the diet of people suffering from allergies (Høst, 2002; Viñas et al., 2014). For these all reasons, the consumption of sheep and goat milk continues to grow.

Goat and sheep milk is constantly compared to cow's milk, whose representation in the total consumption of milk is still dominant.

An evaluation of goat and sheep milk in comparison with cow milk from the point of view of macro- and microelements contents is not uniform. While Gajewska et al. (1997) consider the nutrient composition of cow and goat milk being comparable, according to broad monograph devoted to sheep and goats (Park et al., 2007), sheep and goat milk has around 0.9%, respectively 0.8% total minerals (ash) compared to 0.7% in cow milk. Overall, goat and sheep milk have more Ca, P and Cl, and less Na and S than cow milk. Higher amount of these all elements was found in sheep milk than in goat milk (Park et al., 2007).The content of K in goat milk is higher than in cow milk, on the contrary the content of K in sheep milk is lower than in cow milk. The content of Fe, Cu, and Zn is comparable in all three kind of milk.

Relatively high content of macro- and microelement in sheep milk in comparison with cow milk was found by Hampel et al. (2004) that gave the following values: Ca 2.07 g $kg^{-1}$, Mg 0.24 g $kg^{-1}$, K 1.64 g $kg^{-1}$, P 1.50 g $kg^{-1}$, Fe 0.51 mg $kg^{-1}$, Cu 0.09 mg $kg^{-1}$, Zn 5.10 mg $kg^{-1}$. In goat milk were determined higher amounts of all elements except Fe and Cu than in sheep milk, which reported Kondyli et al. (2007). These authors reported mean mineral content of raw goat milk of the indigenous Greek breed the widespread breed in Greece, the first among European countries in goat population, 1.30 g $kg^{-1}$ for Ca, 0.16 g $kg^{-1}$ for Mg, 1.50 g $kg^{-1}$ for K, 0.59 g $kg^{-1}$ for Na, 0.98 g $kg^{-1}$ for P, 0.60 mg $kg^{-1}$for Fe, 3.70 mg $kg^{-1}$ for Zn and 0.80 mg $kg^{-1}$for Cu.

The composition of the milk produced by small ruminants depends on the breed, feeding, lactation state, individual animal, status of udder health and other environmental conditions (Antunovic et al., 2001; Aganga et al., 2002; Morand-Fehr et al., 2007; Park et al., 2007; Sanz Ceballos et al., 2009; Zervas & Tsiplakou, 2011).

There are not many studies focused at the investigation of mineral content in sheep and goat milk not only in the Czech Republic, especially in real life. These studies also do not mostly distinguish between different breeds.

The aim of this study was to determine and compare the content of sodium, potassium, calcium, magnesium, zinc, copper, lead, and cadmium in sheep and goat milk of different breed from 9 private farms in the Czech Republic.

## MATERIALS AND METHODS

### Experimental material

Goat and sheep pooled milk samples were obtained from 9 different farms (F1-F9) of central, south and east Bohemia. The basis of feed on all the farms was the pasture ad libitum, replenished mainly by hay and silage. Mineral licks were also added. Water was available at all times.

**Individual farm characteristic. Farm F1**: family farm, herd of 100 heads, breed White short haired goat. Feeding: full-day pasture, hay, silage, pressed barley, mineral licks BIOSAXON. **Farm F2:** small family farm, herd of 50 heads, breed Brown short haired goat. Feeding: full-day pasture, hay, silage, pressed barley, branches of pine, oak,

beech, birch, pine branches dominance, mineral licks ALMAGEROL and salt licks. **Farm F3:** small family farm, herd of 40 heads, breed Anglo-Nubian goat. Feeding: full-day pasture, hay, silage, pressed barley, oats. Mineral licks MILLAPHOS Z-V. **Farm F4:** small family farm, herd of 65 heads, breed Lacaune sheep. Feeding: full-day pasture, hay, silage, pressed grains, molasses. Mineral licks SANO. **Farm F5:** big commercially farm, herd of 380 heads of East Friesian sheep, 330 heads of White short haired goat, and 120 heads of Brown short haired goat. Feeding: full-day pasture, hay, silage, pressed grains, corn. Mineral licks: RUMIHERB, NATURMIX. **Farm F6:** big family farm, herd of 130 heads, breed Romanov sheep. Feeding: full-day pasture, hay, alfalfa silage, scrap lupine, pressed grains. Mineral licks: MILLAPHOS and BIOSAXON. **Farm F7:** small family farm, herd of 18 head breed Brown short haired goat. Feeding: full-day pasture, hay, silage, and oat. Mineral licks Schauman – LECKSTEN. **Farm F8:** family farm, herd of 85 heads, breed Lacaune sheep. Feeding: full-day pasture, hay, silage, pressed barley, corn and wheat. Mineral licks SANO. **Farm F9:** small family farm, herd of 30 heads, breed East Friesian sheep. Feeding: full-day pasture, hay, silage, pressed barley, mineral licks RUMIHERB, NATUMIX.

**Sampling and chemical analysis**

**Sampling.** Pool samples of goat and sheep milk were collected repeatedly once a month (April – September) during lactation in the years 2011–2013. Unfortunately, a complete set of samples from all of the farms failed to provide (numbers of analyzed samples are presented in Table 1–6. Statistical analysis was performed for the corresponding set of samples). Samples were collected to the clean, plastic sampling flasks of 100 ml volume, cooled on 4–6 °C, transferred in thermo boxes to the lab and without added preservatives stored at -20 °C in the freezer until analysis. For the determination of Ca, Cd, Cu, K, Mg, Na, Pb, and Zn in goat and sheep milk, aliquots of frozen milk samples (50 ml) were lyophilized using a LYOVAC GT 2 (LEYBOLD-HERAEUS, GmbH, Germany) and then approx. 0.8 g of lyophilized milk was mineralized by dry ashing (Mader et al., 1997; Mader et al., 1998). Analyses were carried out in triplicate.

**Analysis.** Concentrations of K, Na, Ca, Mg, and Zn in the digests were determined by flame atomic absorption spectrometry (FAAS) using a Varian SpectrAA 110 instrument (Varian, Inc., Mulgrave, Victoria, Agilent Technologies Inc., Palo Alto, CA, USA) in an acetylene-air flame at wavelengths 766.5 nm (K), 589.0 nm (Na), 422.7 nm (Ca), 285.2 nm (Mg) and 213.9 nm (Zn), respectively. The widths of spectral intervals were 1 nm (K, Na and Zn) and 0.5 nm (Ca and Mg) respectively. During the measurement of Zn and Mg the background was corrected by a deuterium lamp. In the determination of Ca and Mg, 1% solution of lanthanum nitrate was added as a releasing agent. SIPS (Sample Introduction Pump System) was used for the creation of calibration dependence.

Concentrations of Cd, Cu and Pb in the digests were measured by electrothermal atomic absorption spectrometry (ETAAS) using a Varian AA 280Z (Varian, Belrose, Australia) with graphite tube atomizer GTA 120 and PSD 120 programmable sample dispenser. Wavelengths for individual metals were 228.8 nm (Cd), 324.8 nm (Cu) and 283.3 nm (Pb), respectively. Detailed temperature programs for the determination of Cd, Cu and Pb in milk are described in the Technical Report (Mader et al., 2000).

Standard solutions ASTASOL (Analytika, CR) of Ca, Cd, Cu, K, Mg, Na, Pb and Zn were used in the preparation of a calibration curves for the measurements. Concentration of the standards is 1 g $l^{-1}$. The background of laboratory and used chemicals was monitored by analysis of 14.2% blanks prepared under the same conditions, but without samples, and experimental data were corrected by mean concentration of the elements in blanks, and compared with detection limit (mean ± 3SD of blanks) which were 0.348 mg $l^{-1}$ for Ca, 0.007 mg $l^{-1}$ for Mg, 0.023 mg $l^{-1}$ for Zn, 0.007 mg $l^{-1}$ for K, 0.039 mg $l^{-1}$ for Na, 0.07 μg $l^{-1}$ for Cd, 0.70 μg $l^{-1}$ for Cu, and 0.21 μg $l^{-1}$ for Pb. The quality of analytical data was assessed by simultaneous analysis of certified reference material CRM 063R (Skim milk powder) (3.3% of all the samples). Analytical data obtained for all determined elements were found to be within the confidence interval given by the producer of the CRM for each element.

**Statistical evaluation.** Experimentally obtained data were statistically evaluated by ANOVA method of one and two factor analysis of variance separately for each element; the SAS computer program, version 9.1 (StatSoft, 2011) at the level of significance $P < 0.05$ was used.

## RESULTS AND DISCUSSION

### Monitoring of risk elements Cd and Pb in sheep and goat milk

The contents of the risk elements of Cd and Pb in the sheep and goat milk were established only in the years 2011–2012. 75.8% of the analyses on the contents of the Cd and 66.7% of the analyses on the contents of the Pb were below the limit of detection (0.177 and 0.520 μg $kg^{-1}$, respectively). Other determined values of these two contaminants were lower than maximal tolerable amount (MTA) according to regulation No. 298/1997 Coll. of the Ministry of Health of the Czech Republic given for cow milk (Cd – 10 μg $kg^{-1}$, Pb – 20 μg $kg^{-1}$), which is stringent than the later regulation No. 305/2004 Sb. and sets down the hygienic limit not only for Cd but also for Pb in milk. The highest content of Cd (3.13 μg $kg^{-1}$ d.w.) was determined in sheep milk (Lacaune breed) and the highest content of Pb (7.3 μg $kg^{-1}$ d.w.) was determined in goat milk (White short haired breed). For these reasons, the analysis of these risk elements was not carried out in the following year 2013. The fact that all levels of contaminating chemical elements measured are below the hygienic limits (regulation No. 298/1997 Sb.)) is very positive. Hejtmánková et al. (2002) reported the maximum determined content in raw goat milk 1 μg $kg^{-1}$ for Cd, and 26.5 μg $kg^{-1}$ for Pb. Higher content of Cd and Pb also determined Coni et al. (1996) and Rodriguez et al. (1999). In agreement with recent still lower content of Cd in milk mentioned Elmastas et al. (2005) in goat milk the value 0.085 μg $l^{-1}$.

### Monitoring of nutritional elements K, Ca, Na, Mg, Zn, and Cu in sheep and goat milk

Average values and the range of the determined contents (2011–2013) of selected macro– and microelements for individual farms are summarized in Table 1–6. The values are related to lyophilized milk powder. The content of macro-elements potassium and sodium in milk was determined only in years 2012 and 2013. The contents of each element in the whole of the reference period (2011–2013) moved in a relatively wide

range. Multiple differences in content of some elements in milk are given also by Aganga et al. (2002) and Mayer & Fiechter (2012).

Determined levels of Ca in goat and sheep lyophilized milk powder varied from 1.40 to 8.08 g $kg^{-1}$ and from 1.69 to 9.13 g $kg^{-1}$, respectively. The average content of Ca in goat lyophilized milk powder was 4.99 ± 2.49 g $kg^{-1}$, respectively 5.27 ± 2.25 g $kg^{-1}$ in sheep lyophilized milk powder.

Determined contents of Ca in the milk of small ruminants occur on the lower border of the listed values are extremely low. Low is also average content of Ca as in goat milk, so in sheep milk. Only Khan et al. (2006) shows such low levels of Ca in the milk. These authors indicate 551 ± 12.9 mg $l^{-1}$ in sheep milk in the winter period. Only the maximum values of the Ca content in goat and sheep milk are approaching the values of Ca content in small ruminant milk reported by some authors. Trancoso et al. (2010) reported 10.9 ± 0.8 g $kg^{-1}$ d.w in goat milk, Khan et al. (2006) determined 701 ± 4.1 mg $l^{-1}$ in goat milk in winter period, respectively 961 ± 16.9 mg $l^{-1}$ in summer period, and 900 ± 12.5 mg $l^{-1}$ in sheep milk in summer period. The highest contents of Ca in sheep milk 2.42 g $l^{-1}$ mentioned Mayer & Fiechter (2012), 207 ± 18 mg 100 $g^{-1}$ determined Hampel et al. (2004) and Aganga et al. (2002), 1.397 g 100 $g^{-1}$. The highest content of Ca in goat milk is also given by Aganga et al. (2002), that is 1.097 mg 100 $g^{-1}$. Most of the authors are given the content of Ca in milk of small ruminants in range 1–2 g $kg^{-1}$ (Hejtmánková et al., 2002; Kondyli et al., 2007; Sanz-Ceballos et al., 2009; Kedzierska-Matysek et al., 2013.). In accordance with Park et al. (2007), Mayer & Fiechter (2012) and Aganga et al. (2002) the slightly higher content of Ca was found in sheep milk than in goat milk.

**Table 1.** Calcium content (g $kg^{-1}$) in milk of small ruminants

| Farm | Breed | Year | n | N | Range | Average | Median | St.dev. |
|---|---|---|---|---|---|---|---|---|
| F1 | White short haired goat | 2011 | 12 | 100 | 3.10–4.90 | 3.98 | 3.95 | 0.66 |
| F1 | White short haired goat | 2012 | 18 | 100 | 4.31–7.85 | 6.69 | 7.65 | 1.45 |
| F2 | Brown short haired goat | 2011 | 18 | 50 | 2.50–4.60 | 3.58 | 3.60 | 0.81 |
| F2 | Brown short haired goat | 2012 | 18 | 50 | 5.83–7.53 | 6.83 | 7.02 | 0.58 |
| F3 | Anglo-Nubian goat | 2011 | 18 | 40 | 3.90–5.40 | 4.65 | 4.75 | 0.55 |
| F3 | Anglo-Nubian goat | 2012 | 18 | 40 | 7.15–8.08 | 7.65 | 7.68 | 0.38 |
| F4 | Lacaune sheep | 2012 | 18 | 65 | 5.72–9.13 | 7.57 | 7.89 | 1.17 |
| F5 | East Friesian sheep | 2012 | 18 | 380 | 5.82–8.53 | 7.39 | 7.46 | 0.84 |
| F5 | Brown short haired goat | 2013 | 15 | 120 | 1.79–5.17 | 3.02 | 2.71 | 1.16 |
| F5 | White short haired goat | 2013 | 15 | 330 | 1.40–2.69 | 2.29 | 2.38 | 0.46 |
| F6 | Romanov sheep | 2011 | 18 | 130 | 3.30–5.10 | 4.35 | 4.70 | 0.72 |
| F6 | Romanov sheep | 2012 | 18 | 130 | 4.79–8.66 | 6.17 | 5.90 | 1.21 |
| F7 | Brown short haired goat | 2013 | 15 | 18 | 1.62–2.66 | 2.22 | 2.26 | 0.33 |
| F8 | Lacaune sheep | 2013 | 15 | 85 | 1.79–3.40 | 2.27 | 2.03 | 0.62 |
| F9 | East Friesian sheep | 2013 | 15 | 30 | 2.52–3.32 | 2.98 | 3.05 | 0.32 |
| Average$^{**}$/ total$^{*}$ of all goats | | | 147$^{*}$ | 848$^{*}$ | | 4.55$^{**}$ | 4.67$^{**}$ | 0.71$^{**}$ |
| Average$^{**}$/ total$^{*}$ of all sheep | | | 102$^{*}$ | 820$^{*}$ | | 5.12$^{**}$ | 5.17$^{**}$ | 0.81$^{**}$ |

n – number of samples;
N – number of heads of herd on farm.

Determined levels of Mg in goat lyophilized milk powder were in range 0.16–1.42 g $kg^{-1}$, respectively 0.21–1.36 g $kg^{-1}$ in sheep lyophilized milk powder. The average

content of Mg in goat lyophilized milk powder was 0.94 ± 0.36 g kg$^{-1}$, respectively 0.91 ± 0.31 g kg$^{-1}$ in sheep lyophilized milk powder.

Average content of Mg in goat milk determined in this study is consistent with the values reported by Trancoso et al. (2010), and Khan et al. (2006), lower than the content given by Aganga et al. (2002), Park et al. (2007), Kondyli et al. (2007), Mayer & Fiechter (2012) and Kedzierska-Matysek et al. (2013) On the contrary, lower content of magnesium (752–757 mg kg$^{-1}$ d.w.) in goat milk mentioned Coni et al. (1996). Average content of Mg in sheep milk determined in this study is consistent with the values reported by Khan et al. (2006), lower than the content given by Aganga et al. (2002), Hampel et al. (2004), Park et al. (2007), Mayer & Fiechter (2012). On the other hand, lower content of magnesium (588–653 mg kg$^{-1}$ d.w.) in sheep milk mentioned repeatedly Coni et al. (1996).

**Table 2.** Magnesium content (g kg$^{-1}$) in milk of small ruminants

| Farm | Breed | Year | n | N | Range | Average | Median | St.dev. |
|---|---|---|---|---|---|---|---|---|
| F1 | White short haired goat | 2011 | 12 | 100 | 0.16–0.48 | 0.32 | 0.31 | 0.12 |
| F1 | White short haired goat | 2012 | 18 | 100 | 0.70–1.11 | 0.89 | 0.87 | 0.14 |
| F2 | Brown short haired goat | 2011 | 18 | 50 | 0.42–0.76 | 0.55 | 0.52 | 0.13 |
| F2 | Brown short haired goat | 2012 | 18 | 50 | 0.95–1.20 | 1.08 | 1.11 | 0.10 |
| F3 | Anglo-Nubian goat | 2011 | 18 | 40 | 0.35–0.64 | 0.54 | 0.56 | 0.10 |
| F3 | Anglo-Nubian goat | 2012 | 18 | 40 | 1.10–1.39 | 1.24 | 1.24 | 0.10 |
| F4 | Lacaune sheep | 2012 | 18 | 65 | 0.72–1.08 | 0.92 | 0.93 | 0.12 |
| F5 | East Friesian sheep | 2012 | 18 | 380 | 0.92–1.33 | 1.15 | 1.15 | 0.12 |
| F5 | Brown short haired goat | 2013 | 15 | 120 | 1.16–1.42 | 1.32 | 1.39 | 0.10 |
| F5 | White short haired goat | 2013 | 15 | 330 | 1.21–1.41 | 1.31 | 1.32 | 0.07 |
| F6 | Romanov sheep | 2011 | 18 | 130 | 0.21–0.49 | 0.36 | 0.35 | 0.09 |
| F6 | Romanov sheep | 2012 | 18 | 130 | 0.71–1.13 | 0.89 | 0.89 | 0.14 |
| F7 | Brown short haired goat | 2013 | 15 | 18 | 1.01–1.27 | 1.15 | 1.18 | 0.10 |
| F8 | Lacaune sheep | 2013 | 15 | 85 | 0.83–1.21 | 1.02 | 1.07 | 0.14 |
| F9 | East Friesian sheep | 2013 | 15 | 30 | 1.11–1.36 | 1.19 | 1.15 | 0.10 |
| Average$^{**}$/ total$^{*}$ of all goats | | | 147$^{*}$ | 848$^{*}$ | | 0.93$^{**}$ | 0.94$^{**}$ | 0.11$^{**}$ |
| Average$^{**}$/ total$^{*}$ of all sheep | | | 102$^{*}$ | 820$^{*}$ | | 0.92$^{**}$ | 0.92$^{**}$ | 0.12$^{**}$ |

n – number of samples;
N – number of heads of herd on farm.

Determined levels of K. in goat lyophilized milk powder ranged from 8.16 to 31.10 g kg$^{-1}$, the average content was 18.15 ± 5.03 g kg$^{-1}$. In sheep lyophilized milk powder were recorded levels of K 3.53–11.90 g kg$^{-1}$. The mean level was 7.48 ± 2.59 g kg$^{-1}$. Average content of K in goat milk determined in this study is consistent with the values reported by Kondyli et al. (2007), Park et al. (2007), and Kedzierska-Matysek et al. (2013). Higher value of K content 4.87 g kg$^{-1}$ in raw goat milk determined Aganga et al. (2002). On the contrary, lower average content of potassium in goat milk 12.20 ± 2.00 g kg$^{-1}$ d.w. determined Trancoso et al. (2010), and Khan et al. (2006). The average content of K in sheep milk determined in this study is lower than value reported by Aganga et al. (2002), Hampel et al. (2004), Park et al. (2007) and Mayer & Fiechter (2012). On the contrary, Khan et al. (2006) reported lower content of K (1.079–1.166 mg l$^{-1}$) in sheep milk than it is given in this study. In this study statistically higher ($P < 0.05$) content of potassium was determined in goat milk in comparison with sheep

milk. Higher content of potassium in goat milk than in sheep milk also reported Aganga et al. (2002), Park et al. (2007) and Mayer & Fiechter (2012). By contrast, Khan et al. (2006) determined higher content of potassium in sheep milk than in goat milk (1.122 mg $l^{-1}$ vs 480 mg $l^{-1}$).

**Table 3.** Potassium content (g $kg^{-1}$) in milk of small ruminants

| Farm | Breed | Year | n | N | Range | Average | Median | St.dev. |
|---|---|---|---|---|---|---|---|---|
| F1 | White short haired goat | 2012 | 18 | 100 | 13.21–17.75 | 15.44 | 16.31 | 1.91 |
| F2 | Brown short haired goat | 2012 | 18 | 50 | 8.16–15.32 | 13.50 | 14.57 | 2.50 |
| F3 | Anglo-Nubian goat | 2012 | 18 | 40 | 12.32–17.20 | 14.04 | 12.72 | 2.04 |
| F4 | Lacaune sheep | 2012 | 18 | 65 | 4.17–8.00 | 6.16 | 6.10 | 1.46 |
| F5 | East Friesian sheep | 2012 | 18 | 380 | 5.11–9.73 | 6.84 | 6.74 | 1.54 |
| F5 | Brown short haired goat | 2013 | 15 | 120 | 19.23–22.39 | 21.32 | 21.55 | 1.15 |
| F5 | White short haired goat | 2013 | 15 | 330 | 19.27–31.11 | 24.20 | 22.48 | 4.18 |
| F6 | Romanov sheep | 2012 | 18 | 130 | 3.53–6.26 | 4.77 | 4.50 | 0.90 |
| F7 | Brown short haired goat | 2013 | 15 | 18 | 20.42–25.86 | 22.72 | 23.23 | 1.98 |
| F8 | Lacaune sheep | 2013 | 15 | 85 | 7.87–11.90 | 9.72 | 9.35 | 1.35 |
| F9 | East Friesian sheep | 2013 | 15 | 30 | 10.33–11.65 | 10.87 | 10.83 | 0.44 |
| Average$^{**}$/ total$^{*}$ of all goats | | | 99$^{*}$ | 658$^{*}$ | | 18.54$^{**}$ | 18.48$^{**}$ | 2.21$^{**}$ |
| Average$^{**}$/ total$^{*}$ of all sheep | | | 84$^{*}$ | 690$^{*}$ | | 7.67$^{*}$ | 6.25$^{**}$ | 0.12$^{**}$ |

n – number of samples;
N – number of heads of herd on farm.

Determined levels of Na in goat and sheep lyophilized milk powder varied from 0.72 to 5.43 g $kg^{-1}$ and from 0.605 to 5.05 g $kg^{-1,}$ respectively. The average content of Na in goat and sheep lyophilized milk powder was 2.33 ± 1.51 g $kg^{-1}$ and 1.78 ± 0.13 g $kg^{-1}$, respectively. Average content of Na in goat milk determined in this study is similar to the values (2.83 ± 0.4 g $kg^{-1}$ d.w.) reported by Trancoso et al. (2010) and 0.27 g $kg^{-1}$ reported by Aganga et al. (2002) and other authors (Mayer & Fiechter, 2012; Kedzierska-Matysek et al., 2013). Slightly higher content of Na mentioned Khan et al. (2006), Park et al. (2007), and especially Kondyli at al. (2007). Average content of Na in sheep milk determined in this study is lower than other values given in the literature (Khan et al., 2006; Park et al., 2007; Aganga et al., 2002; Hampel et al., 2004; Mayer & Fiechter, 2012). In summary, it can be said that the content of Na in small ruminant milk was relatively low in this study, but it was moving in a larger range from 0.07–0.17 in goat milk, respectively 0.13–0.33 in sheep milk. Even though the ratio in sheep milk is higher than in goat milk, average Na/K value in sheep milk 0.23 (0.12 in goat milk) is consistent with the ratio (0.23) reported by Trancoso et al. (2010) and lower than reported by Kondyli et al. (2007), Park et al. (2007) and Raynal-Ljutovac et al. (2008). This low Na/K ratio might be of interest from the human nutrition point of view, especially for people suffering from high blood pressure or under dialysis (Trancoso et al., 2010).

**Table 4.** Sodium content (g $kg^{-1}$) in milk of small ruminants

| Farm | Breed | Year | n | N | Range | Average | Median | St.dev. |
|---|---|---|---|---|---|---|---|---|
| F1 | White short haired goat | 2012 | 18 | 100 | 0.74–1.27 | 1.02 | 0.99 | 0.18 |
| F2 | Brown short haired goat | 2012 | 18 | 50 | 0.72–1.04 | 0.95 | 0.99 | 0.11 |
| F3 | Anglo-Nubian goat | 2012 | 18 | 40 | 0.83–1.49 | 1.08 | 1.02 | 0.22 |
| F4 | Lacaune sheep | 2012 | 18 | 65 | 0.89–1.28 | 1.04 | 1.02 | 0.14 |
| F5 | East Friesian sheep | 2012 | 18 | 380 | 0.71–1.03 | 0.88 | 0.92 | 0.12 |
| F5 | Brown short haired goat | 2013 | 15 | 120 | 3.05–4.54 | 3.81 | 3.78 | 0.48 |
| F5 | White short haired goat | 2013 | 15 | 330 | 3.46–5.43 | 4.00 | 3.71 | 0.73 |
| F6 | Romanov sheep | 2012 | 18 | 130 | 0.65–0.93 | 0.82 | 0.84 | 0.10 |
| F7 | Brown short haired goat | 2013 | 15 | 18 | 3.48–4.25 | 3.88 | 4.05 | 0.32 |
| F8 | Lacaune sheep | 2013 | 15 | 85 | 1.64–4.31 | 3.11 | 3.17 | 0.86 |
| F9 | East Friesian sheep | 2013 | 15 | 30 | 2.81–5.05 | 3.56 | 3.15 | 0.83 |
| Average$^{**}$/ total$^{*}$ of all goats | | | 99$^{*}$ | 658$^{*}$ | | 2.46$^{**}$ | 2.42$^{**}$ | 0.34$^{**}$ |
| Average$^{**}$/ total$^{*}$ of all sheep | | | 84$^{*}$ | 690$^{*}$ | | 1.88$^{**}$ | 1.82$^{**}$ | 0.41$^{**}$ |

n – number of samples;
N – number of heads of herd on farm.

Determined levels of Zn in goat lyophilized milk powder were in range 7.59–44.1 mg $kg^{-1}$, respectively 13.7–34.3 mg $kg^{-1}$ in sheep lyophilized milk powder. The average content of Zn in goat lyophilized milk powder was 26.5 ± 7.24 mg $kg^{-1}$, respectively 24.2 ± 4.94 mg $kg^{-1}$ in sheep lyophilized milk powder. Average content of Zn in goat milk determined in this study is nearly identical to the value (26.00 ± 4.90 g $kg^{-1}$ d.w.) reported Trancoso et al. (2010) in the Saanen goat milk, and the value 27.00 mg $kg^{-1}$ d.w. is given by Haenlein & Anke (2011). Slightly higher value of Zn content reported also Kedzierska-Matysek et al. (2013). The contents of Zn in goat milk above 5.0 g $kg^{-1}$ reported Hejtmánková et al. (2002), Elmastas et al., (2005) and Park et al. (2007). Contrarily lower values (16.80–19.30 mg $kg^{-1}$ d.w.) were reported by Coni et al. (1996). The Zn content in sheep milk described in the literature is different. Average content of Zn in sheep milk determined in this study is nearly identical to the interval of Zn content (21.20–2.60 mg $kg^{-1}$ d.w.) reported by Coni et al. (1996). The contents of Zn in sheep milk above 5.0 g $kg^{-1}$ reported Aganga et al. (2002), Hampel et al. (2004) and Park et al. (2007). The highest content of Zn in sheep milk (10.40 ± 0.01 mg $l^{-1}$) mentioned Elmastas et al. (2005). On the contrary, only 0.56 ± 0.06 mg $l^{-1}$ in sheep milk determined Khan et al. (2006) in summer period in semiarid region of Pakistan, respectively 1.29 ± 0.05 mg $l^{-1}$ in winter period.

Determined levels of Cu in goat lyophilized milk powder ranged from 0.21 to 1.46 mg $kg^{-1}$, the average content was 0.57 ± 0.31 mg $kg^{-1}$. In sheep lyophilized milk powder were recorded levels of Cu 0.15–2.10 mg $kg^{-1}$. The mean level was 0.60 ± 0.47 mg $kg^{-1}$ Average content of Cu in goat milk determined in this study corresponds to the value 0.04–0.08 mg $kg^{-1}$ in raw White short haired goat milk determined in two herds in the Czech Republic (Hejtmánková et al., 2002). It is also similar to the content of Cu in goat milk reported by Coni et al. (1996), Elmastas et al. (2005), Trancoso et al. (2010), Haenlein & Anke (2011), Kedzierska-Matysek et al. (2013). Higher contents of Cu in milk are given by Sanz Ceballos (2009), Khan et al. (2006), Kondyli et al. (2007), Park et al. (2007), and Aganga et al. (2002). The contents of Cu in raw goat milk are 0.40 mg $kg^{-1}$, 0.30 mg $kg^{-1}$, 0.40–1.10 g.$l^{-1}$, 0.5 mg $kg^{-1}$ and

1.007–2.007 mg $kg^{-1}$ respectively. Average content of Cu in sheep milk determined in this study is similar to the range of Cu content (0.453–0.784 mg $kg^{-1}$ d.w.) reported by Coni et al. (1996) in sheep milk. Slightly higher value of Cu in sheep milk reported Hampel et al. (2004) and Elmastas et al. (2005). Park et al. (2007) in raw milk mentioned the value 0.40 mg $kg^{-1}$ and Aganga et al. (2002) even the value 2.007 mg $kg^{-1}$.

**Table 5.** Zinc content (mg $kg^{-1}$) in milk of small ruminants

| Farm | Breed | Year | n | N | Range | Average | Median | St.dev. |
|---|---|---|---|---|---|---|---|---|
| F1 | White short haired goat | 2011 | 12 | 100 | 17.70–29.70 | 21.70 | 19.60 | 4.73 |
| F1 | White short haired goat | 2012 | 18 | 100 | 14.92–27.62 | 22.52 | 23.74 | 5.05 |
| F2 | Brown short haired goat | 2011 | 18 | 50 | 24.60–44.10 | 29.20 | 26.65 | 6.79 |
| F2 | Brown short haired goat | 2012 | 18 | 50 | 28.19–39.31 | 32.67 | 32.27 | 3.85 |
| F3 | Anglo-Nubian goat | 2011 | 18 | 40 | 25.00–38.40 | 32.28 | 32.20 | 4.58 |
| F3 | Anglo-Nubian goat | 2012 | 18 | 40 | 27.41–38.90 | 31.54 | 30.82 | 3.76 |
| F4 | Lacaune sheep | 2012 | 18 | 65 | 17.71–34.27 | 25.86 | 27.29 | 5.80 |
| F5 | East Friesian sheep | 2012 | 18 | 380 | 24.26–31.51 | 27.15 | 26.55 | 2.48 |
| F5 | Brown short haired goat | 2013 | 15 | 120 | 18.37–30.93 | 23.39 | 21.77 | 4.34 |
| F5 | White short haired goat | 2013 | 15 | 330 | 7.59–27.67 | 18.27 | 21.77 | 7.22 |
| F6 | Romanov sheep | 2011 | 18 | 130 | 20.70–30.75 | 26.41 | 27.00 | 3.33 |
| F6 | Romanov sheep | 2012 | 18 | 130 | 13.69–26.08 | 19.85 | 19.05 | 4.58 |
| F7 | Brown short haired goat | 2013 | 15 | 18 | 15.54–30.00 | 23.07 | 23.03 | 4.63 |
| F8 | Lacaune sheep | 2013 | 15 | 85 | 16.57–24.33 | 20.33 | 19.99 | 2.48 |
| F9 | East Friesian sheep | 2013 | 15 | 30 | 20.18–29.44 | 25.25 | 26.36 | 3.22 |
| Average$^{**}$/ total$^{*}$ of all goats | | | 147$^{*}$ | 848$^{*}$ | | 26.07$^{**}$ | 25.76$^{**}$ | 4.99$^{**}$ |
| Average$^{**}$/ total$^{*}$ of all sheep | | | 102$^{*}$ | 820$^{*}$ | | 24.14$^{**}$ | 24.37$^{**}$ | 3.65$^{**}$ |

n – number of samples;
N – number of heads of herd on farm.

According to former regulation No. 298/1997 Coll. of the Ministry of Health of the Czech Republic valid for cow milk the maximal tolerable amount for Cu was 0.40 mg $kg^{-1}$ respectively 10.0 mg $kg^{-1}$ for Zn. From this point of view, the relatively low contents of Cu in both goat and sheep milk determined in this study seems to be positive and also the content of Zn in milk meets the former strict rules.

To determine the influence of breed on the mineral contents in milk of small ruminants, the milk of White short haired goat and Brown short haired goat coming from the same farm (F5) in the year 2013 was used (Table 1–6). No statistical difference ($P < 0.05$) between the two goat breeds was found.

To monitor the effect of a specific character of individual farms, goat milk of the same breed from different farms was used, specifically Brown short haired goat (farms F7, F5) (Table 1–6). No statistical difference ($P < 0.05$) between these two farms was found. Slightly higher content of monitored minerals were found in Brown short haired goat milk bred in farm F7.

To determine the influence of the year the milk samples from three goat breeds, namely White short haired goat (farm F1), Brown short haired goat (farm F2), and Anglo-Nubian goat (farm F3) and one sheep breed, namely Romanov sheep (farm F6), were collected in the years 2011–2012. It was found that the year has statistically significant ($P < 0.05$) influence on the content of Ca and Mg in milk of small ruminants. The contents of these elements were higher both in goat and sheep milk in the year 2012.

**Table 6.** Cooper content (mg $kg^{-1}$) in milk of small ruminants

| Farm | Breed | Year | n | N | Range | Average | Median | St.dev. |
|---|---|---|---|---|---|---|---|---|
| F1 | White short haired goat | 2011 | 12 | 100 | 17.70–29.70 | 0.39 | 0.39 | 0.08 |
| F1 | White short haired goat | 2012 | 18 | 100 | 14.92–27.62 | 0.34 | 0.35 | 0.05 |
| F2 | Brown short haired goat | 2011 | 18 | 50 | 24.60–44.10 | 0.27 | 0.27 | 0.04 |
| F2 | Brown short haired goat | 2012 | 18 | 50 | 28.19–39.31 | 0.43 | 0.51 | 0.14 |
| F3 | Anglo-Nubian goat | 2011 | 18 | 40 | 25.00–38.40 | 0.58 | 0.44 | 0.30 |
| F3 | Anglo-Nubian goat | 2012 | 18 | 40 | 27.41–38.90 | 0.43 | 0.45 | 0.03 |
| F4 | Lacaune sheep | 2012 | 18 | 65 | 17.71–34.27 | 0.25 | 0.25 | 0.06 |
| F5 | East Friesian sheep | 2012 | 18 | 380 | 24.26–31.51 | 0.53 | 0.35 | 0.31 |
| F5 | Brown short haired goat | 2013 | 15 | 120 | 18.37–30.93 | 0.87 | 0.99 | 0.23 |
| F5 | White short haired goat | 2013 | 15 | 330 | 7.59–27.67 | 1.07 | 1.01 | 0.20 |
| F6 | Romanov sheep | 2011 | 18 | 130 | 20.70–30.75 | 0.31 | 0.32 | 0.09 |
| F6 | Romanov sheep | 2012 | 18 | 130 | 13.69–26.08 | 0.41 | 0.27 | 0.30 |
| F7 | Brown short haired goat | 2013 | 15 | 18 | 15.54–30.00 | 0.89 | 0.88 | 0.07 |
| F8 | Lacaune sheep | 2013 | 15 | 85 | 16.57–24.33 | 1.10 | 0.90 | 0.50 |
| F9 | East Friesian sheep | 2013 | 15 | 30 | 20.18–29.44 | 1.15 | 1.11 | 0.29 |
| Average$^{**}$/ total$^{*}$ of all goats | | | 147$^{*}$ | 848$^{*}$ | | 0.53$^{**}$ | 0.59$^{**}$ | 0.13$^{**}$ |
| Average$^{**}$/ total$^{*}$ of all sheep | | | 102$^{*}$ | 820$^{*}$ | | 0.63$^{**}$ | 0.53$^{**}$ | 0.26$^{**}$ |

n – number of samples;
N – number of heads of herd on farm.

**Changes during lactation period**

The influence of the lactation period on content of minerals in sheep and goat milk was also monitored. The samples of milk were taken once a month during lactation (April – September) from all farms. The contents of all followed minerals in milk samples from each farm collected during the lactation period were very variable. Variability ranged from 4.05% (K, F9, East Friesian sheep, 2013) to 73.2% (Cu, F6, Romanov sheep, 2012.). The highest average variability of the content of all measured minerals during the lactation period was observed in the year 2013 (22.5%). The average variability observed in the years 2011 and 2012 was very similar 17.4% respectively 18.4%. Slightly higher variability was observed in the sheep milk than in goat milk (20.8% vs 17.4%). As regards the evaluation of the whole study period it could be said that the contents of measured minerals mostly varied significantly ($P < 0.5$) during lactation period.

According Aganga et al. (2002), Hejtmánková et al. (2002), and Kondyli et al. (2007) Ca and Cu showed significantly higher content at the beginning of the lactation period. In this study, the higher content of Ca at the beginning of the lactation period was not confirmed unlike the Cu content, which was mostly the highest at the beginning of the lactation period, especially in the sheep milk. With some deviation the increasing content of Mg in milk of small ruminants according to previous study (Hejtmánková et al., 2002) was observed. Generally, in accordance with Hejtmánková et al. (2002) it is not possible to find any direct relationship between the content of studied elements and the date of sampling.

## CONCLUSION

The contents of risk elements Cd and Pb in milk from all tested farms were very low and met the requirements for hygienic limits according to Regulation No. 298/1997. The levels of Ca, Cu, K, Na, Mg, and Zn in both goat and sheep milk were variable not only during the lactation period, but also during the entire study. The contents of determined elements in this study were average or rather below average in the comparison with the contents of these elements in milk of small ruminants reported in literature. The highest average variability of the content of all measured minerals during the lactation period was observed in the year 2013 (22.5%). An important factor of variability apart different lactation stage proved to be a year, which may be related to the quality of feed and pasture, which is associated with fluctuations in vegetation and climate conditions. The year had significant influence on the content of Ca and Mg in milk of small ruminants, but it is not possible to find any direct relationship between the content of studied elements and the date of sampling. Generally, due to the great variability of elements in milk of small ruminants the differences in the mineral composition between goat and sheep milk were not demonstrated, with the exception of the potassium content.

Significantly higher content of potassium was determined in goat milk than in sheep milk. It is not possible to decide whether statistically different contents of Ca, Cu, Na, Mg and Zn in the milk of small ruminants detected in the same year are the result of a different breed or reflect the specificity of the individual farms.

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# Partial Purification of β–glucosidase enzyme from soybean (Glycine max) and determination of inhibitory effects two quercetin derivatives on enzyme activity

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**Abstract.** Glucosidases are enzymes that catalyze the hydrolysis of the glycosidic linkage of glycosides, leading to the formation of a sugar hemiacetal or hemiketal and the corresponding free aglycon. Activity of glucosidases is crucial for several biochemical processes. Thus, discovery of new glucosidase inhibitors is crucially important owing to potential therapeutic applications of this enzyme in the treatment of diabetes, human immunodeficiency virus infection, metastatic cancer, lysosomal storage disease etc. In the current study, inhibitory potential of 'quercetin' and its isomeric form 'morin hydrate' on the activity of β-glucosidase enzyme, present in the extract of soybean (*Glycine max* L.) seeds, were investigated. The compounds exhibited moderate inhibitory action in low milimolar concentrations. $I_{50}$ values were calculated as 0.188 and 0.138 mM for quercetin and morin hydrate, respectively. The results have confirmed that these compounds can be used as leads for designations of novel glucosidase inhibitors which would be used in medicinal biotechnology and food science and technology.

**Key words:** beta–glucosidase, inhibition, quercetin, morin hydrate, soybean.

## INTRODUCTION

Glucosidases (glycoside hydrolase) are enzymes that catalyse the hydrolysis of glycosidic bonds to form monosaccharides and oligosaccharides and are involved in rearrangement of glycoproteins, glycoconjugates and polysaccharides.

β-glucosidases fall into GH1, GH3, GH5, GH 9 and GH30 families of glycoside hydrolases. Among these, the family containing the largest number of characterized β-glucosidase is GH1 (Niemeyer, 1988; Henrissat, 1991; Henrissat & Davies, 1997). In terms of diversity, β-glucosidases exhibit their biological functions most extensively in plants. It was shown that about 40 GH1 β-glucosidases are expressed in a typical plant (Xu et al., 2004; Opassiri et al., 2006).

β-glucosidases have become the focus of many studies due to their key roles in many biological and biotechnological processes such as growth and development, defense and signaling mechanisms, biomass conversion, nutrient detoxification and nutritional quality. β-glucosidases are of great importance especially for the biomass conversion process. β-glucans including cellulose are globally the most abundant renewable biomass resources and β-glucosidase is the key element for conversion of β-glucans (Gilbert et al., 2008). Some other features of β-glucosidases make them useful for various industrial fields; such as improvement of nutritional quality, flavour and

stability in food sector (Opassiri et al., 2006; Nguyen et al., 2010; Chandra et al., 2013; Souza et al., 2014) bioavailability enhancement in pharmaceutical industry (Kim et al., 2013; Handa et al., 2014), as well as synthesis of certain oligosaccharides and glycosides as food supplements through their ability of catalysing reverse hydrolysis and transglycosylation reactions (Sa´nchez-Pe´rez et al., 2008; Pal et al., 2010; He et al., 2013).

Quercetin is a well known and remarkable flavonoid with regard to its biological protective activities and one of the most potent antioxidants among polyphenols, which is found in many herbal sources including apple, citrus, red grape, onion, tea, etc (Fig. 1) (Formica & Regelson, 1995; Rice–Evans et al., 1997; Prior, 2003). Quercetin is also known to posses antiviral, antibacterial, anticarcinogenic and antiinflammatory effects, and several isoforms of quercetin like aglycone and rutin glycosides in different doses are commercially available as supplements for supportive treatment of many diseases and disorders (Formica & Regelson, 1995; Di Carlo et al., 1999; Harborne & Williams, 2000).

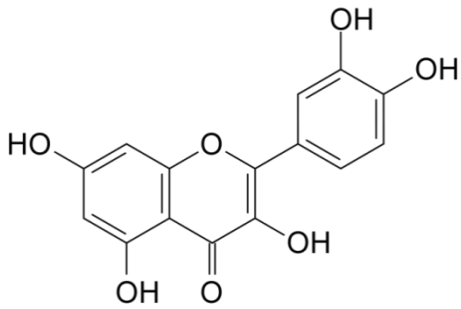


**Figure 1.** Structural formula of quercetin.

Morin hydrate is another attractive bioflavonoid, which is a polyphenolic compund in yellow crystalline structure, found in white mulberry, almond, sweet chestnut and some other fruits (Fig. 2). Morin hydrate serves a variety of pharmacological activities including free radical scavenging, anti–inflammatory effect, protection against DNA damage and low density lipoprotein oxidation, anticancer properties, making it beneficial for therapeutic applications of diabetes, cardiovascular and neurodegenerative diseases (Gopal, 2013).

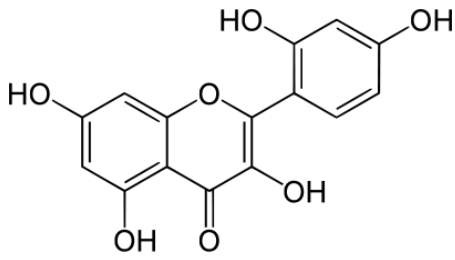


**Figure 2.** Structural formula of morin hydrate.

The objective of this study is to determine the potential effects of mentioned flavonoids, quercetin and morin hydrate, on soybean β-glucosidase enzyme as novel inhibitors. There has been a growing interest in manipulating the activity of glucosidase enzymes, in consequence of their important roles in many metabolic processes. Several kinds of chemically synthesized or naturally isolated glucosidase inhibitors are of great concern (Berecibar et al., 1999; Asano, 2003; Kim et al., 2006; Li et al., 2006; Pandey et al., 2006, Khalaf et al., 2015) as valuable biochemical tools and potential therapeutic agents, making substantial contributes to reveal the activity of the enzyme, understanding structures of potential inhibitors and discovery of compounds with a variety of promising applications.

## MATERIALS AND METHODS

### Materials

All chemicals used in soybean extraction, enzyme inhibition and purification steps, including p-Nitrophenyl β-D-glucopyranoside (p-NPG), dipotassium phosphate, quercetin, morin hydrate, sodium chloride, ammonium sulphate, ethanol, hydrogen chloride, sodium hydroxide were obtained from Sigma-Aldrich Co.

### Plant material

Raw seeds of a soybean (*Glycine max* L.) genotype supplied by Ondokuz Mayıs University, Faculty of Agriculture were used for extraction.

### Extraction and *in vitro* glucosidase inhibition assay

Soybean seeds were powdered in liquid nitrogen, following a physical fractionation. B-glucosidase activity was determined pursuing the procedure described by Ribeiro et al. (2006), with slight modifications. 100 mg of ground soybean sample was kept in a 0.05M phosphate buffer (pH 4.5), containing 0.1M NaCl, for 1 h at 4 °C. Following a centrifugation step, supernatant was filtered and directly used for further analyses.

For β-glucosidase activity, p-nitrophenyl-β-D-glucopiranoside (p-NPG) was used as substrate. 500 µl of p-NPG in 0.1M phosphate buffer (pH 5.0) was transferred to cuvette and pre-heated in a water bath for 5 min at 30 °C. Enzymatic reaction was initiated by adding 125 µl of supernatant containing β-glucosidase into the cuvette and final volume was completed to 1ml with distilled water; and then the absorbance value at the beginning of the reaction was read in a spectrophotometer at 420 nm. Samples in the cuvette were again put in the water bath for 10 min, at 30 °C. Final absorbance value was read in the spectrophotometer after this duration, to be compared with the initial value and was recorded as 100% control activity in the absence of an inhibitor. For inhibition assays, 1mM solutions of quercetin and morin hydrate were also included in the cuvette mixture. Different volumes of these compunds were added into the mixture in order to increase the inhibitor concentartion gradually; and equal volumes of water was diminished from the mixture. A graphic consisting of percent activity versus natural molecule concentration was drawn for each of the compounds. Cuvette contents used in the β-glucosidase activity tests are shown in Tables 1, 2.

**Table 1.** Details of cuvette components used to determine $I_{50}$ values of quercetin flavonoid on soybean β-glucosidase

| p-NPG/ $K_2HPO_4$ (pH 5) (µl) | Soybean extract (µl) | $H_2O$ (µl) | Inhibitor (Quercetin) (µl) | Inhibitor concentration (mM) | Total volume (ml) |
|---|---|---|---|---|---|
| 500 | 125 | 375 | – | – | 1 |
| 500 | 125 | 340 | 20 | 0.020 | 1 |
| 500 | 125 | 325 | 35 | 0.035 | 1 |
| 500 | 125 | 300 | 60 | 0.060 | 1 |
| 500 | 125 | 275 | 150 | 0.150 | 1 |
| 500 | 125 | 225 | 200 | 0.200 | 1 |

**Table 2.** Details of cuvette components used to determine $I_{50}$ values of morin hydrate flavonoid on soybean β-glucosidase

| p-NPG/ $K_2HPO_4$ (pH 5) (µl) | Soybean extract (µl) | $H_2O$ (µl) | Inhibitor (Quercetin) (µl) | Inhibitor concentration (mM) | Total volume (ml) |
|---|---|---|---|---|---|
| 500 | 125 | 375 | – | – | 1 |
| 500 | 125 | 355 | 20 | 0.020 | 1 |
| 500 | 125 | 340 | 35 | 0.035 | 1 |
| 500 | 125 | 305 | 70 | 0.070 | 1 |
| 500 | 125 | 245 | 130 | 0.130 | 1 |
| 500 | 125 | 225 | 150 | 0.150 | 1 |

**Partial purification of the enzyme**

Soybean extract was precipitated with ammonium sulfate, where the total concentration varided between 10–100%. Precipitates of every stage were collected by centrifugation at 11,000 rpm for 30 min and redissolved in 0.05M $K_2HPO_4$ buffer (pH 4.5). All the process was carried out on ice. The highest enzyme activity value for soybean β-glucosidase was observed at 30–40% concentration interval.

## RESULTS AND DISCUSSION

**Effects of flavonoid compounds on soybean β-glucosidase**

The potential inhibitor compounds quercetin and morin hydrate inhibited β-glucosidase at millimolar levels. $I_{50}$ values of flavonoids quercetin and morin hydrate were calculated as 0.188 and 0.138, respectively. Observed percent activity values of enzyme in response to different concentrations of both compounds are presented in Tables 3, 4, and Figs 3, 4.

**Table 3.** Effect of flavonoid quercetin on soybean β-glucosidase enzyme activity

| Concentration (mM) | Activity % |
|---|---|
| 0.000 | 100.0 |
| 0.020 | 93.8 |
| 0.035 | 82.2 |
| 0.060 | 73.4 |
| 0.150 | 56.0 |
| 0.200 | 48.3 |

**Table 4.** Effect of flavonoid morin hydraye on soybean β-glucosidase enzyme activity

| Concentration (mM) | Activity % |
|---|---|
| 0.000 | 100.0 |
| 0.020 | 92.7 |
| 0.035 | 85.8 |
| 0.070 | 64.0 |
| 0.130 | 54.9 |
| 0.150 | 42.2 |

Activity of glucosidases, including β-glucosidase, fulfils key roles in many biochemical processes. Their functions in the organism are also associated with several diseases and disorders; thus discovery of potential inhibitors of glucosidases as therapeutic agents against diabetes, obesity, viral infections, lysosomal storage diseases and cancer has been a challenging subject (Kordik & Allen, 1999; Platt & Butters, 2000; Lillelund et al., 2002; Papandréou et al., 2002; Gunasekaran et al., 2014).

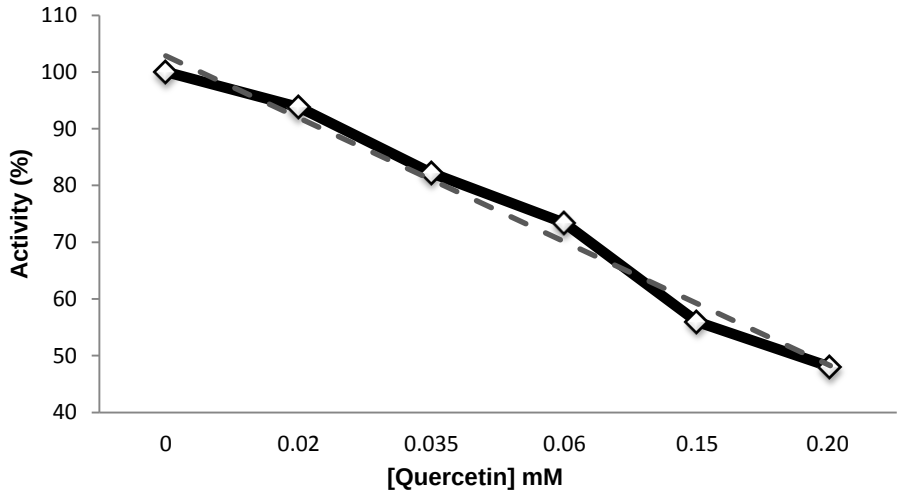


**Figure 3.** Effect of quercetin on soybean β-glucosidase enzyme activity.

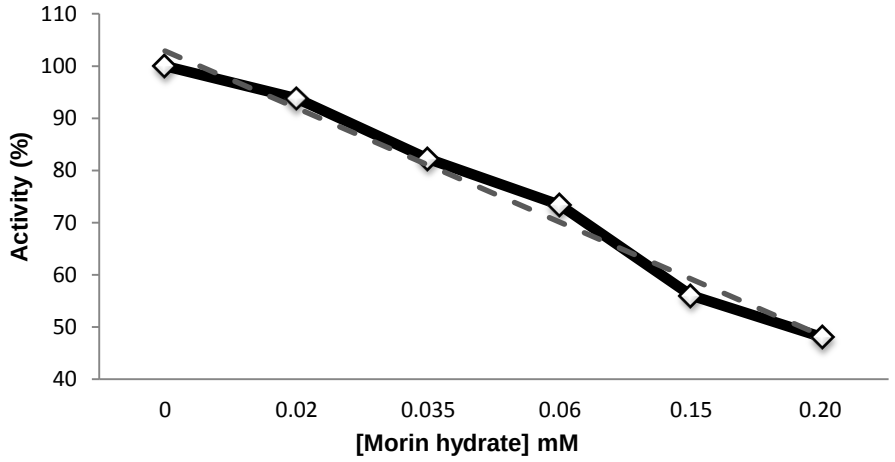


**Figure 4.** Effect of morin hydrate on soybean β-glucosidase enzyme activity.

Altering or blocking particular metabolic activities, glucosidase inhibitors served for understanding functions of glucosidases and enabled novel approaches to be utilized in several fields, other than medicine. For instance, glucosidase inhibitors can also function as agricultural chemicals such as antifungals insecticides (Asano, 2003).

## CONCLUSIONS

Our study aimed to examine the inhibitory potential of two common compounds in nature, flavonoid quercetin and its isomeric form morin hydrate, on soybean β-glucosidase enzyme. The enzyme was partially purified, and both of the natural compounds were able to inhibit the β-glucosidase enzyme within the extract of soybean seeds at low concentrations.

Inhibitors of glucosidases are basically in glycosidic structure. In common to the two flavonoids investigated in this research, compunds which do not apparently bear structural similarities to carbohydrates constitute a new category of inhibitors and understanding of their functions and mechanisms are crucial for providing new approaches in terms of dicovery of new therapeutic agents. Inhibition of key enzymes by natural molecules has been recently the basis of pharmacology, biochemistry and chemistry research. Concerning the therapeutic potential of glucosidase inhibitors, current study provides precious information for further investigations.

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# The influence of heat transfer coefficient on moisture evaporation rate during the cooling of fresh baked white pan bread

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**Abstract.** Cooling rate is a very critical parameter. Low cooling rates can limit production capacity in a bakery, while higher cooling rates can lead to a higher moisture evaporation rate and result in the greater weight loss of the product. The principal objective of this work is to study the effect of heat transfer coefficients on heat and mass transfer processes, which take place in freshly baked white pan bread during its cooling.
The model of bread cooling process is built based on experimental results, Fourier's second law for heat transfer and Fick's second law for mass transfer. The new model allows studying what influence the heat transfer coefficient has on the cooling rate. Several dependencies are revealed and discussed in this article. Several pieces of advice for developing an air distribution system are also provided.

**Key words:** Bread cooling, heat transfer coefficient.

## INTRODUCTION

The increase in the market share of food products, including bakery products, is possible owing to the transition to modern intensive industrial modes of production. Only that way is it possible to ensure the sales of a wide range of quality products in increasing quantities with increasing profitability. Upgrading production with modern technologies and equipment under the conditions of a shortage of resources requires a systematic scientific approach. Attempts of using direct simple solutions are doomed to failure.

Bread production is a laborious and rather time-consuming process, which includes a number of operations. Changing operating parameters takes a long time, which leads to the deterioration of product quality. The cooling of bread to be cut into slices and packaged is an important part of the overall bread making process chain.

In packaging hot products moisture accumulates inside the package, which leads to the wetting of crust, accelerated development of moulds and loss of bread products' appearance and presentation. Moreover, the high-quality cutting of hot bread is associated with certain difficulties. On the other hand, the packaging of entirely cold bread, which has already lost a significant amount of moisture during the cooling process (due to shrinkage), is inappropriate, since in such bread the rate of staling increases significantly. That is why the determination of the optimal cooling period of bakery

products could increase the storage time of packaged bread, while the product will maintain good sensory properties and presentation. The following requirements are applied to products to be packaged: in case of products made of rye and rye-wheat flour weighing 0.7–1 kg, the optimal period of cooling prior to packaging is 90–120 minutes for pan bread and 80–100 min for the hearth products; the optimal duration of cooling for bakery products weighing 0.3–0.5 kg is 60–70 minutes.

The problems related to the cooling of bakery products at bakeries are solved in different ways depending on available funds, production facilities, production volumes, and energy requirements. Technically, the process of cooling bread is usually organised in the following ways:

1. In cooling chambers (on fixed pallets or carts). This method requires large areas. The cooling time is over 3 hours.

2. In vacuum systems using evaporative cooling. The cooling time is less than 0.5 hours. In terms of capital and operating costs this method is the most expensive; the performance of facilities is low.

3. On conveyor lines in tunnel or tower installations. The duration of cooling is about 2 hours.

The advantages of applying spiral conveyor systems have been widely discussed for over 5 years (Pastukhov & Danin, 2011). The problem of cooling bread can be solved using a tower conveyor (Fig. 1). This is done in one of the leading St. Petersburg branch enterprises that brings together leading experts in the field of air conditioning and refrigeration equipment from ITMO University and the Bureau for the Equipment of Air Conditioning and Refrigeration Ltd.

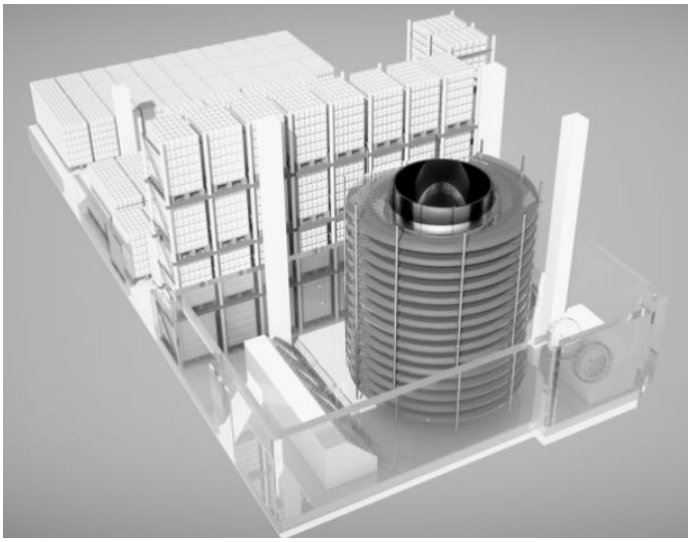

**Figure 1.** 'Tower' conveyor.

There is a computer system for calculating the temperature at the centre of the loaf on the basis of the temperature on its surface (Pastukhov, 2015). The system takes into account the thermal properties (Jarny & Maillet, 1999), shape and mass of the freshly baked product, and can be therefore used for calculating the cooling parameters of

various bakery and other food products (Simpson & Cortes, 2004). The calculations are based on experimental studies (choice of initial and boundary conditions for modelling), literature data (Zueco et al., 2004) and mathematical modelling (Van der Sluis, 1993; Zanoni et al., 1994).

## MATERIALS AND METHODS

Food materials, including bakery products, are complex heterogeneous objects. Knowledge of the thermal characteristics of a product is required for calculating cooling process kinetics. When using thermal characteristics values provided in literature, large errors are possible because the conditions of determining values are often different from operating conditions. The task of calculating the parameters of bakery products' cooling process is a complex problem involving unsteady heat and mass transfer. This study uses the classical methods of calculating unsteady heat transfer for bodies with limited dimensions (a cylinder and a box), such as the regular mode method and elementary heat balance method (Vanichev, 1946).

The calculation results are shown in Figs 2, 3. They allow judging the theoretically possible cooling rate of bread, as well as the influence of the main factors, such as air temperature and heat transfer coefficient (α), on the intensity of heat transfer.

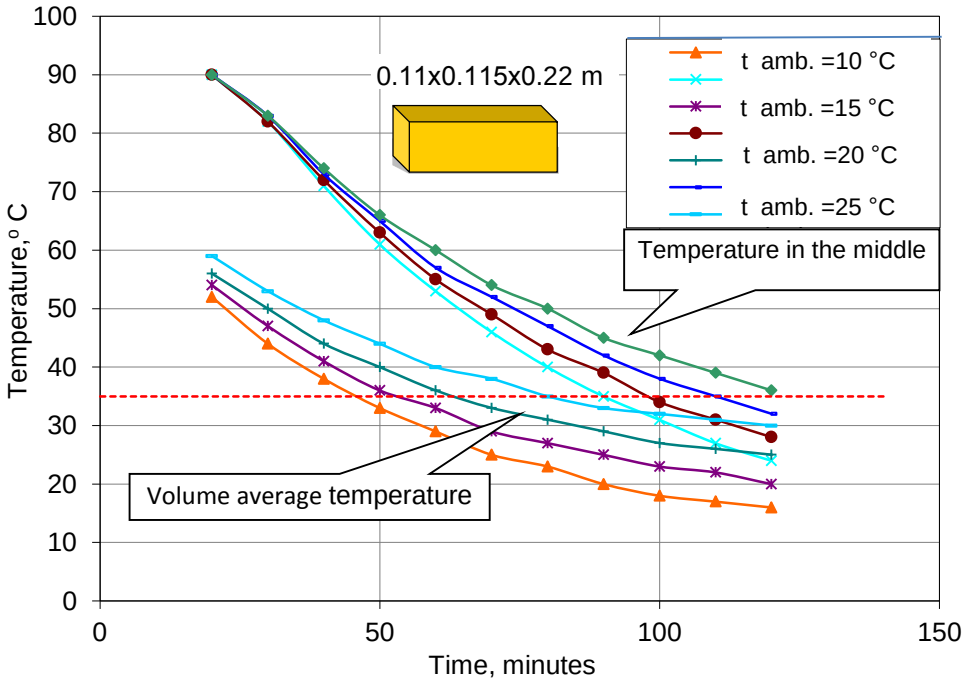


**Figure 2**. Dependence of bread cooling on ambient temperature (lowest heat transfer coefficient on the surface of the loaf is 15 W $(m^2 K)^{-1}$).

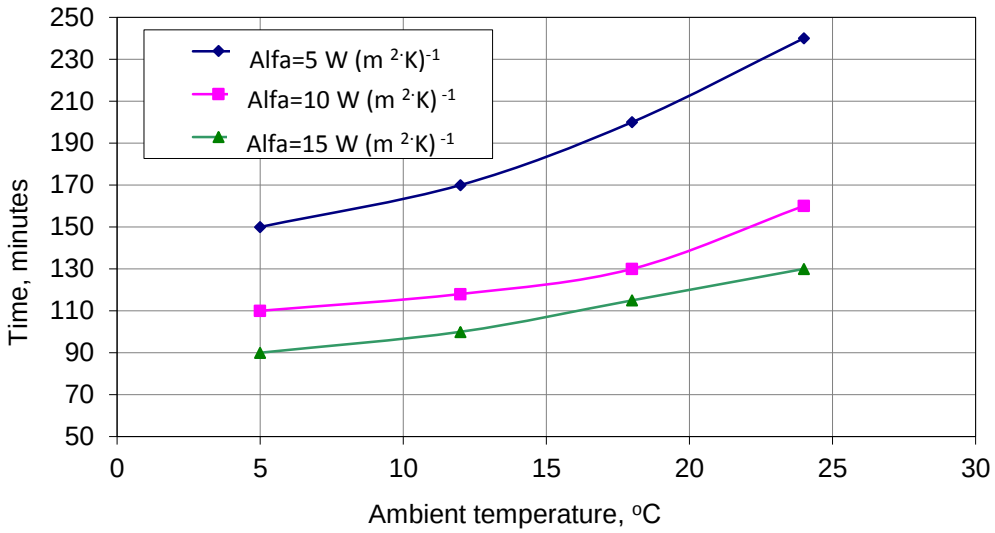


**Figure 3.** Dependence of cooling time on ambient temperature and heat transfer coefficient.

The discrepancies between the cooling time calculations carried out independently with different methods are no more than 10–15%. However, the theoretical data require validation through experiments in all cases.

## RESULTS AND DISCUSSION

It is important to limit the cooling temperature range with the maximal values of heat transfer coefficient on the surface of the bread to achieve minimal temperatures in the cooling tower. The value of the first parameter directly depends on the mobility of ambient air near the bread crust surface. Values of $\alpha = 5$; 10; 15; 20 W $(m^2 K)^{-1}$ correspond to the air speed $\omega = 0.5$; 1.5; 2.5; 3 m $s^{-1}$. The minimum level of air temperature at the exit of air coolers in the standard air handling units of air conditioning systems is 8–10 °C. A further decrease in temperature is problematic due to the necessity to maintain negative temperatures on the heat exchange surface and to organise periodic defrosting. In real conditions currently used at bakery products' production plants the average value of the heat transfer coefficient is no more than 12–15 W $(m^2 K)^{-1}$. In order to increase the value of this parameter it is necessary to develop an air distribution system based on experiments and mathematical simulations.

1. As a result of the preceding discussion, the following conclusions can be drawn: 1. The minimum time of cooling the centre of the bread from 90 °C to 35 °C with an average air temperature of 5 °CC; 10 °CC; 15 °CC; 20 °CC; 25 °CC and an air velocity of $\omega = 1.5$ m $s^{-1}$ ($\alpha = 10$ W $(m^2 K)^{-1}$) near the surface of the bread will be approximately 95; 105; 110; 125; 140 minutes respectively. In this case the mean bulk temperature of 35 °CC may be achieved in 50; 58; 68; 80; 100 minutes respectively.

2. The minimal time of cooling the centre of the bread from 90 °CC to 35 °CC with an average air temperature of 10 °CC; 15 °C; 20 °C; 25 °C and an air velocity of $\omega = 2.5$ m $s^{-1}$ ($\alpha = 15$ W $(m^2 K)^{-1}$) near the surface of the bread will be approximately 90; 98; 110; 125 minutes respectively. In this case the mean bulk temperature of 35 °C can be achieved in 46; 50; 60; 80 minutes respectively.

Obtaining a temperature of 35 °C in the centre of the bread after 1 hour is problematic in the considered conditions.

The actual cooling rate with optimum air distribution is 100 and 110 minutes when the average air temperature in the tower is 10 °C and 15 °C respectively ($\alpha = 10$ W $(m^2 K)^{-1}$). In these cases, the maximum output of bread will decrease from 2,715 kg $h^{-1}$ to 1,629; 1,480 kg $h^{-1}$, and heat generation will be 95; 81 kW (considering shrinkage) respectively.

However, it would be more correct to take the average bulk temperature as a determined value. In this case, it is possible to get the actual cooling time of 70; 80 minutes. In this case the maximum output of bread will decrease from 2,715 kg $h^{-1}$ to 2,327; 2,036 kg $h^{-1}$, and heat generation will be 148; 120 kW (considering shrinkage) respectively. It is necessary to consider heat loss through the protecting constructions of the cooling tower, electric drives, and other unaccounted heat input in the amount of at least 25–30 kW in calculations and the design of the cooling towers.

The above calculations are applied to the most massive volume, and, as a result, the most 'unfavourable' shape (in terms of heat mass transfer) of loaf can be said to be that of the pan bread 'Stolichniy'. The actual cooling rate of hearth loaves can be significantly higher than that of 'Stolichniy' due to their lower weight and size.

An important factor limiting the intensity of heat and mass transfer, and, consequently, the cooling rate of bread, is the optimization of air distribution in the working zone of the cooling tower. The determining factors that influence the rate of heat transfer on the surface of the bread are the velocity of the air coming from the air distributors, angle-of-attack of air flow relative to the axis of the conveyor, air temperature, temperature of the loaf, tightness of the jet by the conveyor elements, etc. The computing package STAR-CD—a specialized complex high-level program certified in accordance with ISO 9001 available from Computational Dynamics Ltd—was used for solving mathematical problems. Efficient algorithm parallelization decisions based on the finite volume method combined with the unique methods of automated partitioning of the flow region can simulate computational fluid dynamics tasks with any degree of complexity. The model of the bread cooling process was built based on experimental results, Fourier's second law for heat transfer and Fick's second law for mass transfer. The model is also based on the relationship between the Biot number and cooling rate during natural or forced cooling that was discussed before (Pastukhov & Danin, 2012). Results of the computational experiment for the determination of the optimum angle-of-attack of air flow by ITMO University, Institute of Refrigeration and Biotechnologies are shown in Fig. 4 and Table 1.

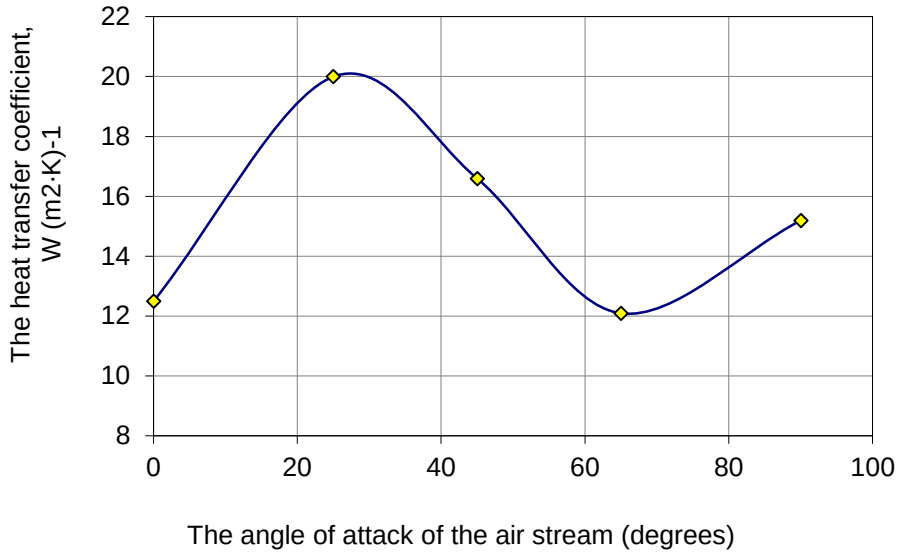


**Figure 4.** Dependence of the effective heat transfer coefficient on the surface of the loaf on the angle-of-attack of the air stream.

**Table 1.** Heat transfer coefficient on the surface of the loaf

| Parameter | | | | | |
|---|---|---|---|---|---|
| angle ° | 0 | 25 | 45 | 65 | 90 |
| air flow rate, m $s^{-1}$ | 2 | 2 | 2 | 2 | 2 |
| air temperature, °C | 7 | 7 | 7 | 7 | 7 |
| heat transfer coefficient, W $(m^2 \cdot K)^{-1}$ | 12.5 | 20 | 16.6 | 12.1 | 15.2 |

## CONCLUSIONS

Results of the theoretical and pilot researches conducted allow drawing the following conclusions:

The maximum heat transfer coefficient (about 20 W $(m^2\ K)^{-1}$) was observed at the angle of 25 (air flow rate 2 m $s^{-1}$). Thus, an adequate air distribution system should be developed to ensure these parameters.

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# Effect of heat treatment at constant 120 °C temperature on the rheological and technological properties of pork

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**Abstract.** The aim of the study was to evaluate the influence of low-temperature heat treatment on the physical and technological properties of pork. The *Longissimus thoracis* muscles from four pigs were used to determine the quality indicators of pork at 24 hours after slaughtering. Meat samples were cooked at constant 120 °C in a cooking bag until the internal temperatures of 62, 67, 72, 77 and 82 °C. Raw meat was the darkest and differed considerably ($P < 0.05$) from the heat-treated meat. The colour values of the heat-treated meat differed slightly among internal temperature phases. The ultimate pH value of raw meat also differed significantly ($P < 0.05$) from that of cooked meat. The pH value of cooked meat varied only within the range of 0.05 units. The electrical conductivity of muscle decreased gradually as the temperature increased. In case of heat-treated meat, the cooking loss increased considerably (from 18.88% to 31.73%) along with the increase in the internal temperature. The Warner-Bratzler shear force value was the highest (38.50 N) in the meat cooked until 77 °C, and the lowest (28.51 N) in that cooked until 67 °C. Strong negative correlation ($P < 0.001$) between electrical conductivity and cooking loss was observed during the heating procedure. Heat treatment can significantly decrease the electrical conductivity and increase the cooking loss of meat. Meat was the toughest when the internal temperature was 77 °C. However, the best rheological properties were observed in the meat cooked until the internal temperature of 72 °C.

**Key words:** pork, *Longissimus thoracis*, temperature, heat treatment, technological properties, rheological parameters.

## INTRODUCTION

The most important indicators of eating quality are the rheological properties of meat, especially tenderness, which can considerably be altered by heat treatment. Cooking is an acknowledged method of making meat products palatable and safe (Thornberg, 2005), defined by Davey & Gilbert (1974) as the heating of meat to a sufficiently high temperature to denature proteins. During heating denaturation of meat proteins takes place and meat becomes tougher. Heat treatment has also a considerable effect on meat colour as denaturation of myoglobin changes the colour from red to

brownish. Consumers often estimate the doneness of meat by colour (Mancini & Hunt, 2005). Colour also affects the acceptability of the finished products by consumers (Thornberg, 2005). These qualities can be controlled by selecting an appropriate cooking method, cooking time and temperature.

There are several reports available, which declare that the ultimate internal temperature has a major effect on the rheological and technological properties of meat (Cross et al., 1976; Combes et al., 2004; Barbera & Tassone, 2006; Christensen et al., 2011; Huang et al., 2011; Grujić et al., 2014). However, the cooking methods, heating parameters and internal temperatures vary among studies. An air/steam combined cooking technique was used by Gardes et al. (1995), Vittadini et al. (2005), Danovska-Oziewicz et al. (2007) at 180 or 220 °C. Air/steam cooking at considerably lower temperatures (100 to 140 °C) was carried out by Chivaro et al. (2009). Roasting of meat in an oven has usually been done at lower temperatures, e.g. Lien et al. (2002) roasted pork loin chops at 176.7 °C, while Grujić et al. (2014) used a temperature of 163 ± 2 °C. Cooking at lower temperatures may reduce energy consumption, but the final internal temperature must ensure the (hygienic) safety of meat (Smith & LeBlanc, 1990). In some studies, meat has been heat treated in a water bath (Christensen et al., 2011; Huang et al., 2011; Grujić et al., 2014). Internal temperatures of the samples vary between studies, which must be taken into account when comparing the results.

The aim of the study was to evaluate the influence of heat treatment at a low temperature (120 °C) on the physical and technological properties as well as on the textural parameters of pork.

## MATERIALS AND METHODS

**Sample preparation and heat treatment.** Meat samples (*Longissimus thoracis*) from four randomly selected commercially reared crossbred pigs were used in this study. The pigs were slaughtered at about 6 months of age and 100 kg of live weight in the same slaughterhouse under similar conditions according to European Council Regulation No 1099/2009. Carcasses were cooled for 24 hours, after which the longest spinal muscles (*Longissimus thoracis*) were excised between the 12$^{th}$ *thoracic vertebra* and the 5$^{th}$ *lumbar vertebra* from both sides of each carcass. Muscles were packed into plastic bags and labelled as 1, 2, 3 and 4. Bags were kept in a cooling box during transportation and until the testing.

Raw meat analyses and heat-treatments of the samples were performed immediately upon arrival at the laboratory. Muscles were trimmed of visible fat and connective tissue and cut perpendicularly to muscle fibres into six 30 mm pieces. Five chops were weighed and placed into sealed cooking bags designed for heat treatment (Fig. 1). Each meat sample was supplied with a dual-channel thermocouple to continuously monitor the internal temperatures. Dry heat treatment at 120 °C was used for oven roasting. A total of four series of heat treatment experiments were carried out – one per muscles used in study.

Samples were heated to predetermined internal temperature (62, 67, 72, 77 and 82 °C), taking into account the recommendations of the U.S. Food and Drug Administration (FDA, 2013) according to which fresh pork is safe to eat when cooked to the internal temperature of 63 °C. After heat treatment, samples were cooled to room

temperature and stored in a refrigerator at 4 °C. The pH, colour, electrical conductivity and shear force of the cooked meat samples were measured after being cooled to room temperature.

**Figure 1.** Sealed bags with meat chops in a preheated oven.

**Properties of meat.** Cooking time of the samples from three different muscles used in study was similar. However, cooking time of the samples obtained from the fourth muscle was 6–8 minutes shorter than that of muscle 2, which was caused due to the smaller diameter of the muscle. Samples from, muscle 3 achieved the designated internal temperature 10–13 minutes later than those from muscle 4 (Fig. 2).

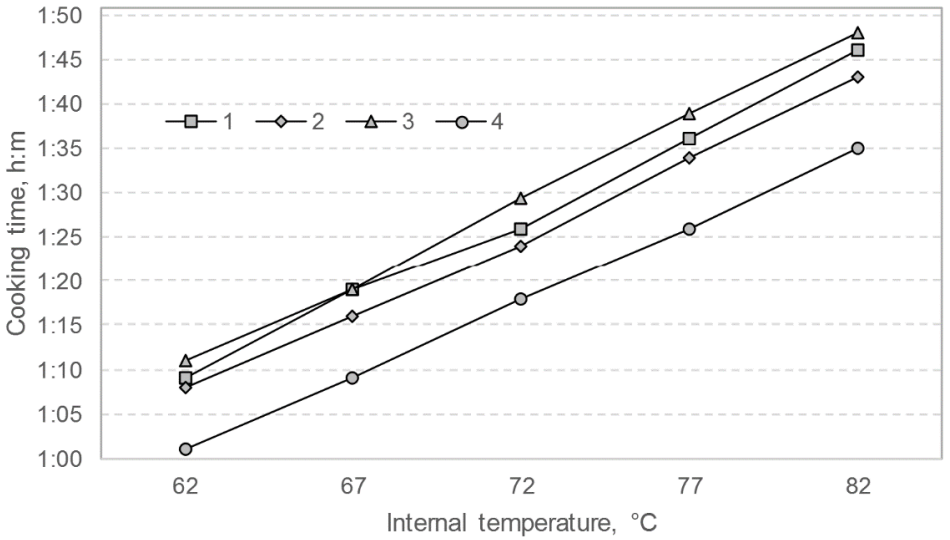


**Figure 2.** Cooking time to the designated internal temperatures of muscle samples 1, 2, 3 and 4.

**Dry matter content and water binding capacity**. The difference between the dry matter content of the raw meat samples did not exceed 1.70%, which indicated that there were no signs of abnormal muscle metabolism (PSE or DFD damage) after slaughtering. However, the water binding capacity of the muscles varied by 15.40%, ranging from 57.60 to 73.00% (Table 1).

**Table 1.** Properties of muscle 1–4 samples

| Trait | Muscle | | | | Average |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | |
| Dry matter content, % | 26.00 | 26.10 | 25.30 | 27.00 | 26.10 |
| Water binding capacity, % | 57.60 | 64.50 | 66.40 | 73.00 | 65.38 |

**Cooking loss.** Cooked samples were removed from bags and cooled until room temperature. Meat chops were wiped dry with blotting paper. Each sample was weighed, and cooking loss (CL) was calculated as follows:

$$CL\ (\%) = \frac{raw\ sample\ weight - cooked\ sample\ weight}{raw\ sample\ weight} \cdot 100 \qquad (1)$$

In addition, the weight of each cooking bag with the liquid released from meat was recorded.

**pH.** Raw and cooked meat pH values were measured at room temperature (~21 °C) using a Testo 205 digital tester (Testo Ltd, Alton, GB). After calibration of the pH meter with standard solutions (pH 4.0 and 7.0) at room temperature, pH was recorded by sticking a probe into the samples. The pH values of raw meat were recorded 24 hours after slaughtering, and those of the heat-treated meat after cooling down the samples.

**Colour.** The colour of each meat sample was measured on the surface of the samples at room temperature (~21 °C) using a digital Opto-Star device (Ingenieurbüro R. Matthäus, Klaus, Germany). Raw meat optimum values are between 60–80 points, but values below 55 points indicate existence of PSE meat and above 85 points to DFD meat (LSZ Boxberg, 2011).

**Electrical conductivity.** The electrical conductivity of the samples was recorded with the LF-Star CPU device (Ingenieurbüro R. Matthäus, Klaus, Germany) by measuring the electric conductance between the two steel electrodes stuck into the meat samples.

**Texture.** The texture was measured in both raw and cooked meat after chilling at 4 °C for 24 hours. A drilling press equipped with a sampling tube was used to obtain 11 mm diameter cores. Ten cores per sample were drilled along the muscle fibre orientation. Each core was sheared once across the centre of the core with a Warner-Bratzler texture analyser TA.XT*Plus* (Stable Micro System Ltd, Godalming, GB) to measure the shear force. Working conditions during the test: blade speed 10 mm $s^{-1}$, maximal load 50 kg, and cutting range 25 mm.

**Statistical analysis.** The data from four randomly selected replicates were analysed using SAS software (SAS Institute Inc., Cary, USA). The two-factor analysis of variance, that included the potential influence of the muscle (1–4), was used to evaluate the effect of cooking time on different characteristics of meat. Additionally, as regards

the meat texture measurement, the interaction between the cooking time and the muscle was tested in ten replications. The differences between individual least square means were estimated using the Tukey's Studentized Range (HSD) test. Pearson correlation analysis was used. A significance level of $P < 0.05$ was chosen.

## RESULTS AND DISCUSSION

**Cooking loss.** Cooking loss is usually defined as the loss of liquid and soluble substances from meat during heat treatment, whereas the main component is water (Heymann et al., 1990). Water is located mostly between muscle fibres and in muscle cells. Heat treatment leads to loss of water due to denaturation of myofibrillar proteins.

The internal temperature of the meat had a significant effect on cooking loss that was 18.88% at 62 °C and 31.73% at 82 °C, whereas the samples lost most of their weight due to the released liquid (18.17 and 30.21%, respectively) (Table 2). An increase in cooking loss was observed during heating, which is consistent with previous studies on pork (Christensen et al., 2011; Huang et al., 2011), beef (Obuz et al., 2004), and rabbit meat (Combes et al., 2004).

The highest weight loss was observed when the internal temperature reached 72 °C. Huang et al. (2011) demonstrated the highest cooking loss (18.80%) at internal temperatures between 60 and 80 °C. The cooking loss of each individual sample was quite similar (se = 0.08%) at 72 °C, whereas the largest differences occurred at 67 °C (1.60%).

**Table 2.** Least square means (± standard error) of meat quality traits estimated in porcine *Longissimus thoracis* at different phases of low-temperature heat treatment

| Trait | Internal temperature, °C | | | | | |
|---|---|---|---|---|---|---|
| | 18 | 62 | 67 | 72 | 77 | 82 |
| Cooking loss, % | | 18.88 ± 0.92$^{a}$ | 21.44 ± 1.60$^{ab}$ | 23.20 ± 0.08$^{bc}$ | 27.32 ± 1.16$^{c}$ | 31.73 ± 1.27$^{d}$ |
| Cooking loss as liquid, % | | 18.17 ± 0.86$^{a}$ | 20.07 ± 1.83$^{ab}$ | 22.02 ± 0.28$^{ab}$ | 25.84 ± 1.16$^{bc}$ | 30.21 ± 1.20$^{c}$ |
| Colour (Opto-Star, points) | 70.93 ± 2.41$^{a}$ | 15.48 ± 0.87$^{b}$ | 15.65 ± 1.26$^{b}$ | 11.95 ± 1.13$^{b}$ | 13.30 ± 0.83$^{b}$ | 15.23 ± 1.28$^{b}$ |
| Ultimate pH | 5.38 ± 0.03$^{a}$ | 5.72 ± 0.01$^{b}$ | 5.74 ± 0.03$^{b}$ | 5.75 ± 0.02$^{b}$ | 5.74 ± 0.02$^{b}$ | 5.77 ± 0.03$^{b}$ |
| Electrical conductivity, mS | 11.94 ± 0.85$^{a}$ | 9.33 ± 0.27$^{b}$ | 8.38 ± 0.24$^{bc}$ | 7.88 ± 0.31$^{bc}$ | 6.95 ± 0.31$^{cd}$ | 5.55 ± 0.40$^{d}$ |
| Warner-Bratzler shear force, N | 22.80 ± 0.75$^{a}$ | 33.25 ± 0.74$^{b}$ | 28.51 ± 0.74$^{c}$ | 33.41 ± 0.85$^{b}$ | 38.50 ± 0.84$^{d}$ | 32.83 ± 0.65$^{b}$ |

The letters ‘a’, ‘b’, ‘c’ and ‘d’ refer to the significance of difference between the values in row at the level of at least 95%.

**Colour.** Heat treatment had a visible influence on the colour of meat, which was caused by denaturation of myoglobin. Raw meat was significantly darker (70.93 points) compared to the samples cooked to the internal temperature of 62 °C (15.48 points). However, increase in the internal temperature from 62 to 82 °C did not produce a noticeable effect on meat colour (Table 2). Similar results were obtained also by Huang et al. (2011), who demonstrated that heating the samples from the initial internal

temperature of 25 to 50 °C in a water bath significantly increased the L*, a* and b* values (Minolta CR-400 Chroma Meter). They found no changes in colour when increasing the temperature from 60 to 80 °C, except for the a* value, which decreased significantly. Lien et al. (2002), however, observed some changes in the colour (measured both visually and instrumentally) of the meat cooked from 62.8 to 82.2 °C. Howe et al. (1982) reported a decrease in the visually estimated colour values when pork chops reached the internal temperature of 70 °C.

**Ultimate pH.** The lowest ultimate pH (5.38) was measured in raw meat. Although the ultimate pH values of cooked meat were only by 0.34–0.39 units higher, this difference proved to be significant ($P < 0.05$) (Table 2). Dal Bosco et al. (2001) and Huang et al. (2011) also found, that heat treatment of raw meat resulted in the increase in pH. Huang et al. (2011) explained that the changes in the pH values of meat during heating were caused by changes in the balance of acid-base groups. In agreement with Huang et al. (2011), the present study showed that the ultimate pH of meat samples did not differ significantly ($P > 0.5$) at different internal temperature levels (62 to 82 °C) through the temperature phases.

**Electrical conductivity.** Electrical conductivity of meat is related to the water content of the muscle tissue. The cooking loss experiments showed that due to the loss of water during heat treatment the samples lost their ability to conduct electricity ($r = –0.941$) (Table 3). Significant difference ($P < 0.05$) was detected between the electrical conductivity of raw meat (11.94 mS) and that of the samples cooked to the predetermined internal temperature levels. Samples cooked to the highest internal temperature (82 °C) proved to be of considerably lower conductivity (5.55 mS) than those cooked to other temperatures (Table 2). The biggest difference in the electrical conductivity values (2.61 mS) at different predetermined temperature levels was found between 18 and 62 °C.

**Texture.** Several authors (Bouton & Harris, 1972; Davey & Gilbert, 1974; Huang et al., 2011) have reported that toughening of meat due to heat treatment occurs in the course of the following two temperature phases: up to 60 °C, and between (60)65 and 80 °C. The explanation to this observation is that denaturation of connective tissue and myofibrillar proteins takes place at different cooking phases (temperatures). Furthermore, the method of heat treatment may also considerably affect tenderness (Huang et al., 2011; Grujić et al., 2014).

Heat treatment had a major effect on the texture of meat. The force to shear the samples increased from 22.80 to 33.25 N ($P < 0.05$) as the internal temperature of raw meat was raised to 62 °C. The shear force remained about the same at the internal temperatures of 62, 72 and 82 °C. Considerable decrease in shear force was detected at 67 °C (28.51 N), while the highest value was observed at 77 °C (38.50 N). Grujić et al. (2014) detected no textural changes in cooked meat at 61, 71 and 81 °C, while significant changes took place at 51, 91 and 100 °C. Huang et al. (2011) obtained similar results at different temperature phases (internal temperatures of 25–50 °C and 60–100 °C).

**Correlations of meat quality traits.** A significant ($P < 0.001$) relationship was observed between the cooking loss, the cooking loss due to the loss of liquid, and electrical conductivity (Table 3). The results confirm previous findings that both traits are affected by the water content of meat.

Contrary to Huang et al. (2011) who estimated that cooking loss was moderately or strongly ($P < 0.01$) related to the pH, shear force and colour values, the present study showed weak unreliable correlations between these characteristics. However, moderate ($P > 0.05$) correlation was found between cooking loss and shear force (r = 0.312).

**Table 3.** Pearson correlation coefficients between meat quality traits of the cooked porcine *Longissimus thoracis* muscle

| | Cooking loss | Cooking loss due to loss of liquid | Colour | Ultimate pH | Electrical conductivity |
|---|---|---|---|---|---|
| Cooking loss due to loss of liquid | 0.913*** | | | | |
| Colour | –0.164 | –0.191 | | | |
| Ultimate pH | 0.108 | 0.122 | 0.090 | | |
| Electrical conductivity | –0.941*** | –0.815*** | 0.131 | –0.271 | |
| WBSF | 0.312 | 0.209 | –0.193 | –0.080 | –0.256 |

*** – $P < 0.001$.

## CONCLUSIONS

Heat treatment of meat at a low temperature significantly affected the physical and technological properties as well as the textural parameters of pork. Cooking loss and electrical conductivity of meat varied through all the temperature phases showing a strong negative relationship between these two traits. The latter leads to a conclusion that both traits are affected by the water content of meat. The major changes in meat colour and ultimate pH occurred already at 62 °C, whereas further heating did not considerably alter these qualities. Due to heating the meat got tougher compared to raw meat. Further heat treatment did not reveal any clear trends as regards meat texture changes, since major changes had occurred at lower temperatures. In summary, the study showed that the rheological properties were optimal in case the meat was cooked to 72 °C.

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# Changes in the total phenol content in the industrial potato peel wastes during the storage

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**Abstract.** As a zero value by-product from the economic point of view, potato (*Solanum tuberosum* L.) peel is a good source of phenols. As a manufacturing waste, potato peels are stored at the uncontrolled conditions and are exposed to the fermentative, oxidative, and microbial degradation. The aim of the present study was to determine the phenol degradation dynamics in the stored peels so the maximum storage time could be defined to achieve the efficient phenol extraction. Three different types of samples were prepared by abrasion peeling method and stored at room temperature, in open air, up to six days. Phenol extracts were obtained using ethanol-based solvent. Total phenol content was expressed as a gallic acid equivalent; antiradical activity was measured using the 2,2-diphenyl-1-picrylhydrazylradical. Results revealed that total phenols during the storage are more stable in the larger peel samples that can be stored up to two days without significant changes in the total polyphenol content and antiradical activity. Finely shredded peel demonstrated significant decrease in the total phenol amount and in the antiradical activity already on the second day of the storage. This fact indicated that in the finely shredded peel samples phenols are easily accessible to the oxidative and fermentative processes. It is possible, that after peeling there were big amounts of chlorogenic acid in the samples. When total amount of polyphenols decreased, chlorogenic acid degraded and caffeic acid was released in sufficient amount to hold antiradical activity of the extract on the high level.

**Key words:** antiradical activity, potato peel utilization, phenols.

## INTRODUCTION

Worldwide, potato (*Solanum tuberosum* L.) production is growing annually and it reached 376.5 million tons in 2013 (FOASTAT, 2013). It is one of the most important agricultural crops for human consumption after wheat (*Triticum* L.), rice (*Oryza* L.), and maize (*Zea mays subsp. mays* L.), due to its low cost, low fat and high carbohydrate contents, valuable proteins, fibres, and another compounds. In the developed countries, the biggest part of the harvest is processed and potato waste (PW) amounts can reach up to 40% of the initial product amount depending on the quality of the raw material and processing technology. Because of the high availability of the specialized animal feed and the high water content in the PW, cattle breeders lose their interest in the PW application that results in the utilization problems. At the same time, Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 require minimization of the produced waste amounts and several goals are established by year 2020 (Directive 2008/98/EC, 2008). Biogas production is an alternative way of the PW

recycling, but it leads to a loss of various valuable compounds that can be extracted and applied in the food production.

There are various types of PW depending on the potato processing method (Ahokas et al., 2014) and the most common is potato peels (PP). PP contain high amount of water (83.29 g 100 $g^{-1}$), is a good source of carbohydrates (12.44 g 100 $g^{-1}$), also contain proteins (2.57 g 100 $g^{-1}$), lipids (0.10 g 100 $g^{-1}$), and dietary fibres (2.5 g 100 $g^{-1}$) (USDA, 2015). In addition, PP is a good source of phenolic compounds (up to 977 mg 100 $g^{-1}$ on a dry weight basis in gallic acid equivalents (GAE)) (Makris et al., 2007), which content varies greatly depending on the potato cultivars (Albishi et al., 2013; Murniece et al., 2014).

Many scientific articles are discussing extraction of phenol compounds form the PP (Al-Weshahy & Rao, 2009; Schieber & Saldaña, 2009; Singh & Saldaña, 2011; Luthria, 2012; Wijngaard et al., 2012; Albishi et al., 2013; Cardoso et al., 2013; Amado et al., 2014; Sánchez Maldonado et al., 2014; Sabeena Farvin et al., 2012), possible application of the peel extract in the food production (Mansour & Khalil, 2000; Zia-Ur-Rehman et al., 2004; Koduvayur Habeebullah et al., 2010; Nayak et al., 2011; Pasqualone et al., 2013; Shah et al., 2014), and its effect on the health (Singh & Rajini, 2004; Singh et al., 2008; Singh & Rajini, 2008). In most studies, peel samples were used for the active compound extraction right after peeling. However, it is very interesting to investigate the possibility of industrial potato processing waste accumulation for further processing. Therefore, the aim of the present study was to determine the phenol degradation dynamics in the stored potato peels for establishing the maximum storage time to achieve the efficient phenol extraction.

## MATERIALS AND METHODS

### Chemicals and reagents

Agricultural origin ethanol (96.6%) was purchased from Stumbras (Kaunas, Lithuania), Folin-Ciocalteu phenol reagent, $Na_2CO_3$ and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$, 95%) from Merck (Darmstadt, Germany).

### Sample preparation

Potato tuber samples (*Solanum tuberosum* cv Magdalena, pedigree 82-28.9/15876.41) were received from the State Priekuli Plant Breeding Institute. Cultivar has yellow flesh and red peel.

Based on the previous study of the peel samples from the potato processing facilities in Latvia, three types of samples were prepared using the abrasion peeling method: a) big peel flakes (3–5 cm), b) small peel particles (3–5 mm), and c) finely shredded peel. Samples were stored in the open plastic containers from 1 to 6 days at 20 ± 3 °C in open air without additional aeration. After collection, samples were freeze-dried in the laboratory dryer FT33 (Armfield, UK) up to approximately 5% moisture content and ground in the laboratory mill KN 195 Knifetec (Foss, Denmark) till the fine PP powder was received. Remaining moisture content was determined by gravimetric method by drying the acquired PP powder at 105 °C to a constant weight. PP powder was immediately used for the phenol extraction without any additional storage. The whole experiment was repeated in three consecutive batches.

**Extraction**

Extraction was performed according to the method described by Mane et al. (2015) with some modifications. 20 mL of the ethanol-water solvent (4:1; v/v) was mixed with 2 g of the PP powder. Mixture was placed in the laboratory ultrasonic bath YJ5120–1 (Zhengzhou Henan, China) for 15 minutes at 33 ± 1 °C. Then mixture was centrifuged for 15 min at 3,000 $s^{-1}$ and filtered. Extraction was repeated three times. Combined supernatants were used for the determination of the total phenol content and antioxidant activity.

**Determination of the total phenol content**

Jung et al. (2011) method with slight modifications was applied. Briefly, 200 µL of the extract solution was mixed with 1 mL of distilled water and 100 µL of Folin-Ciocalteu phenol reagent (preciously diluted with distilled water 1:1, v/v). After 5 min, 2 mL of the 10% sodium carbonate solution was added and mixture was allowed to stand at room temperature for 60 min, and then centrifuged at 3,500 $s^{-1}$ for 10 min. The absorbance was measured at 725 nm by laboratory spectrophotometer Jenway 6300 (Stone, United Kingdom). Results were recalculated per dry matter and expressed as GAE.

**Determination of the free radical scavenging activity**

2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) was used for *in vitro* determination of free radical scavenging activity (Yu et al., 2003). 0.5 mL of extract was mixed with 0.004% DPPH$^{\bullet}$ ethanol solution. Mixture was allowed to stand for 30 min in the dark at room temperature. The absorbance was measured at 517 nm by laboratory spectrophotometer Jenway 6300 (Stone, United Kingdom). The percentage of remaining DPPH$^{\bullet}$ was calculated as follows:

$$(\%) = (1 - \frac{A_{sample}}{A_{control}}) \times 100 \quad (1)$$

where: $A_{control}$ – absorbance of the control sample; $A_{sample}$ – absorbance of the analysed sample.

**Statistical methods**

One-way ANOVA ($P \leq 0.05$) and Tukey's test were applied for the statistical analysis of the effect of the storage time on the total phenol content and effect of the storage time on the free radical scavenging activity of the potato peel extracts.

## RESULTS AND DISCUSSION

Results show that during the storage at uncontrolled conditions phenols are more stable in the larger peel samples (large flakes and small particles) than in the finely shredded peel samples.

During the first minutes after peeling, peel samples showed signs of the browning, which indicated a presence of the fermentative reactions. Vamos-Vigyazo (1981) had reported that polyphenol oxidase (PPO) in potatoes catalyzes the conversion of monophenols to *o*-diphenols and *o*-dihydroxyphenols to *o*-quinones. After that, quinine products can polymerize and react with amino acid groups of cellular proteins that will

lead to brown pigment appearing. In addition, Thygesen et al. (1995) showed that the highest activity of the PPO is in the subepidermal or outer cortex layer, in distance of 1.5–2.0 mm from the skin. Exactly the layer that is taken off by abrasion peeling methods that are applied in the Latvian potato processing facilities.

Strong obnoxious smell appeared after the first day of storage in the finely shredded peel samples, that is a sign of putrefaction and microbial degradation. Meanwhile, cell condition could be a reason that larger peel samples had no signs of the microbial spoilage during the tested storage time.

Fig. 1 shows the effect of the storage time on the total phenol content in the samples. No significant difference was observed between large peel flakes and small peel particles. Total phenol amount was stable during the first two days of storage and showed a significant decline on the third, by 40% comparing to the average of the previous days. Meanwhile, finely shredded peal show significant decrease already on the second day of the storage, by 66%. Difference in the peel sample physical conditions and total phenol content indicate that in the phenols in finely shredded peel samples are more easily exposed to the fermentative and microbial degradation, while in the larger peel samples the whole structure of the cell can express a protective effect.

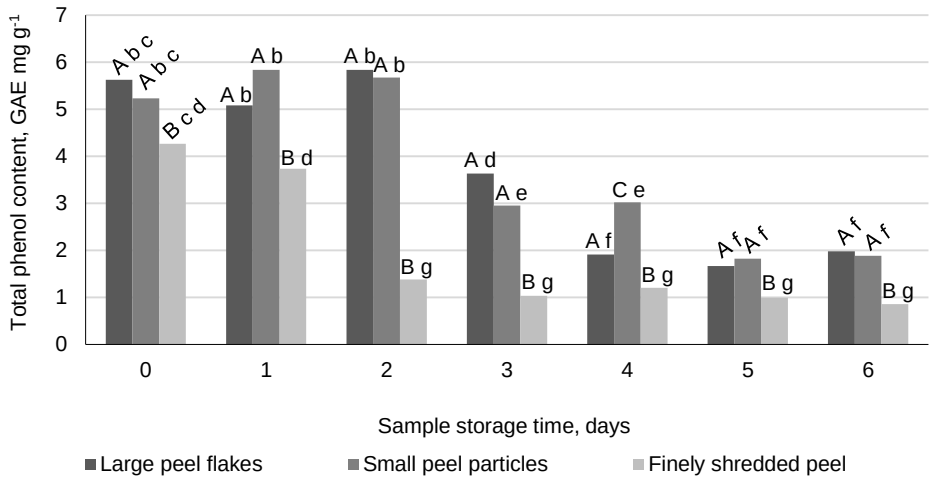


**Figure 1.** Effect of the storage time at uncontrolled conditions on the total phenol content in the potato peel samples. The data is presented as a mean (n = 9). Similar lowercase letters indicate no significant difference among samples ($P \leq 0.05$); similar uppercase letters indicate no significant difference among peel types within each day separately ($P \leq 0.05$).

Fig. 2 shows the effect of the storage time at uncontrolled conditions on the free radical scavenging activity of the potato peel extracts. After the first day of storage all three types of PP samples showed no significant changes in the antiradical activity, but on the second day finely shredded peel had lost 97% of its antiradical activity while activity of the larger peel samples stayed at the previous level. Only on the third day of storage, larger peel samples started to show the first significant decline in its antiradical activity.

It can be observed (Fig. 3) that correlation between total phenol content and antiradical scavenging activity is not linear. It is well known that chlorogenic acid and caffeic acid are two of the main phenols in potatoes (*Solanum tuberosum* L.) and both act as antioxidants (Mattila & Hellström, 2007; Al-Weshahy & Rao, 2009; Koduvayur Habeebullah et al., 2010; Singh & Saldaña, 2011; Wu et al., 2012; Sánchez Maldonado et al., 2014). Chlorogenic acid is an ester of the caffeic and (-)-quinic acid and it is reported that esterification of caffeic acid by a sugar moiety decreases its antioxidant activity (Cuvelier et al., 1992). Studies show that chlorogenic acid is less effective as an antioxidant than caffeic acid in lard and stripped corn oils (Chen & Ho, 1997). At concentrations of 0.5, 1.0 and 2.0 mM, caffeic was more effective against alkoxyl radical scavenging in sunflower oil (Milic et al., 1998) and better inhibited lipid oxidation in fish muscles (Medina et al., 2007). Contrary, it is possible that the binding of the quinic acid to caffeic acid increases antioxidant activity and decreases hydrogen peroxide and DPPH$^{\bullet}$ scavenging activities (Sroka & Cisowski, 2003). Marinova et al. (2009) had found that difference in the antioxidative activity of chlorogenic and caffeic acids during antioxidation of triacylglycerols of sunflower oil strongly depends on their concentrations and at $2.8 \times 10^{-4}$ M both acids show equal effectiveness and strength, but at concentrations above $10 \times 10^{-4}$ M caffeic acid is much more effective and stronger inhibitor. Marinova et al. (2009) explained this phenomenon with two reasons: a) there are specific interactions of the acids and b) the participation of radicals derived from chlorogenic acid on more than one reaction of chain propagation, while the radicals of caffeic acid participate in one reaction.

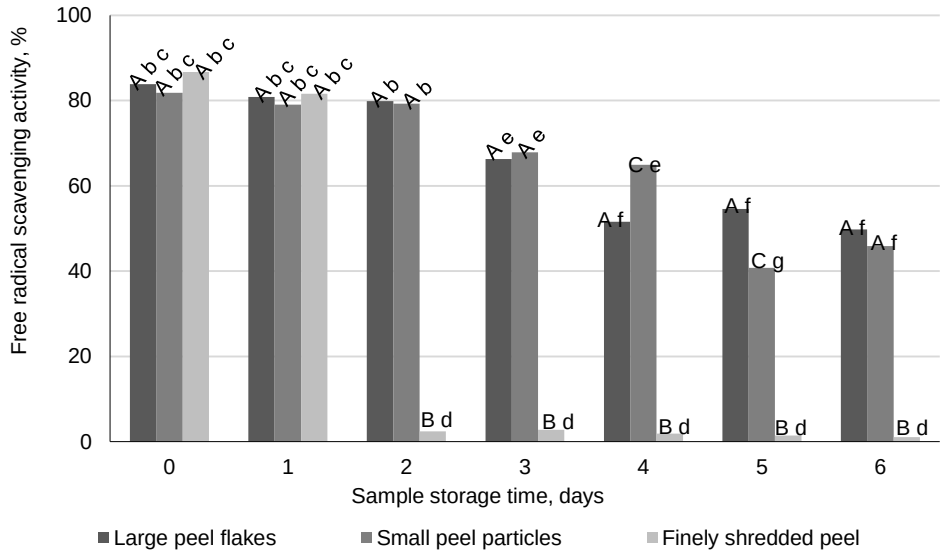


**Figure 2.** Effect of the storage time at uncontrolled conditions on the free radical scavenging activity of the potato peel extracts. The data is presented as a mean (n = 9). Similar lowercase letters indicate no significant difference among samples ($P \leq 0.05$); similar uppercase letters indicate no significant difference among peel types within each day separately ($P \leq 0.05$).

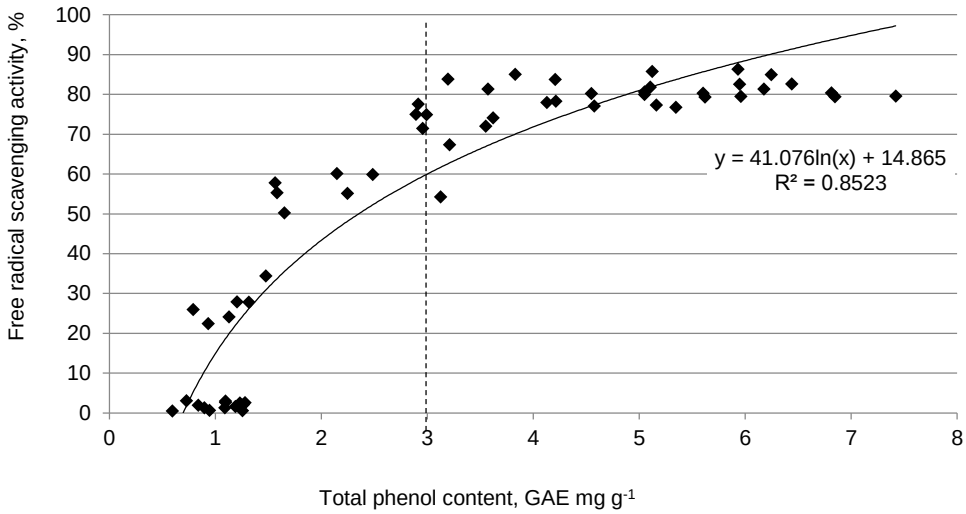


**Figure 3.** Correlation between total phenol content and the free radical scavenging activity.

Usually, chlorogenic acid is most abundant in PP, but when PP are stored at room temperature or in light, chlorogenic acid degrades and transforms into caffeic and quinic acids (Sotillo et al., 1994). Based on the collected data it can be assumed, that after peeling there were big amounts of chlorogenic acid in the samples. When total amount of polyphenols started to decrease, chlorogenic acid degraded and caffeic acid was released in sufficient amount to hold antiradical activity of the extract on the high level till the total phenol content did not decrease below 3.0 mg $g^{-1}$ GAE (Fig. 3) when caffeic acid concentration became too low.

Combining data of total polyphenol content and antiradical activity, it is concluded that larger PP wastes can be stored in the plastic open containers at 20 ± 3 °C in open air without additional aeration for up to two days without significant changes in the total polyphenol amounts and antiradical activity. Finely shredded PP is easily exposed to the microbial and fermentative degradation and should not be stored at the same conditions longer that one day.

## CONCLUSIONS

Results show that larger peel samples, large flakes and small peel particles, can be stored in the plastic open containers at 20 ± 3 °C in open air without additional aeration for up to two days without significant changes in the total polyphenol amount and antiradical activity. Starting with the third day, total phenol amount in the both peel sample types decreased in average by 40%, comparing to the previous day. Finely shredded peel show significant decrease in the total phenol amount, by 66%, and in the antiradical activity, by 97%, already on the second day of the storage. This fact indicates that in the finely shredded peel samples phenols are easily accessible to the oxidative and fermentative processes. Based on the results, if there are no special conditions for the storage of the potato peel wastes at the processing plant, for the efficient phenol extraction purpose it is recommended to store the whole solid peel material, that is not

grinded, no longer than for two days. Meanwhile, finely shredded peel must not be stored more than one day. Acquired results make a basis for the upcoming study on the application of the potato peel wastes and its extracts in the food production.

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# Influence of the packaging material on the quality parameters of tobacco during ageing

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**Abstract.** Tobacco is one of the most extensively studied plant materials in the history. During its production tobacco leaf goes through many different operations from curing (drying) to ageing. Among all of them storage and ageing are very important operations. Properly stored tobacco develops its full flavour, becomes more aromatic and is ready to be sent to a customer for cigarette production. In this work changes in the quality of dried tobacco leafs during ageing (12 and 24 months) in different packaging materials are evaluated. Four samples of the FCV tobacco (Flue-Cured Virginia) grown in Northern Light Soils (NLS) region (India) were analysed. Two different liners inside of C-48 cartons – polyliner and kraft paper – were used for tobacco packaging. Quality evaluation of tobacco samples was done on the basis of analysis of chemical compo-nents (total alkaloids, reducing sugars, volatiles) and colour changing during the ageing process. Organoleptic analyses were performed as a final assessment of tobacco flavour and quality.

**Key worlds:** tobacco, total alkaloids, reducing sugars, volatiles, kraft paper, polyliner.

## INTRODUCTION

Tobacco is one of the most economically significant agricultural crops in the world. The biggest producers and consumers are China, India, Brazil, USA and the European Union. The most widely used types of tobacco are Flue-Cured Virginia (FCV), Burley and Oriental tobacco (Davis, 1999). FCV tobacco is known for its sweet and aromatic taste and is mainly used for cigarette production. The main exporters of FCV tobacco are Brazil and India. After harvesting, tobacco leaf goes through many operations to become the final product. It must be cured (dried) and graded according a complex of physical characteristics. After packaging, tobacco products are stored and aged in warehouses for a period of 6 months to a couple of years. During each operation, quality control of the product is performed checking its physical or chemical characteristics. Tobacco storage and ageing are the last and very important operations before sending tobacco to a customer. Properly stored tobacco develops its full flavour, becomes more aromatic and usually darkens in colour. It is necessary to provide quality control of the packaging tobacco to avoid any mould or pest damage since it may lead

to the loss of the product (Manickavasagan et al., 2007). Tobacco may be packaging with two different types of liners according to customer's request: polyliner or kraft paper. Tobacco stored either with polyliner or with kraft paper liner differs in colour, aroma and taste and physical characteristics (Ming, 2000; Senhofa, 2015). During storage of tobacco in the years 2010–2015 were observed harvest and post-harvest operations and internal and external factors that affect the quality of raw materials and final product.

## MATERIALS AND METHODS

Four samples of the FCV tobacco, grown in Northern Light Soils (NLS) region, harvested and cured in 2011 and 2012, were provided by an unnamed company. The company does not wish to disclose its name. NLS region is located India. The altitude of NLS is in the range of 42 to 90 meters above sea level. The average day temperature during tobacco growing season was in the range of 20 to 34 °C with level of relative humidity of 60–90% and average annual rainfall of 1,231 mm. Soil types range from sandy to sandy loams (89% sand, 4% silt, 7% clay) with pH 5–6. The samples came from equivalent packages from two different customers using two different types of liners inside of C-48 cartons: kraft paper (Fig. 1) and polyliner (Fig. 2). Kraft paper is paper produced from pulp obtained by alkaline (sulphate) method. This paper is stronger than paper made from pulp obtained by acidic (sulphite) method. Polyliner is a packaging material from a vinyl polymer.

The mass of each sample was 500 grams.

**Figure 1.** C-48 cartons with kraft paper (author's archive).


**Figure 2.** C-48 cartons with polyliner (author's archive).

The samples of tobacco harvested in the year 2011have number 1, in the year 2012 number 2. The four samples are named in the following way (Table 1).

**Table. 1** Sample identification

| Sample | Year | Liner | Sample name |
|---|---|---|---|
| 1 | 2011 | Kraft paper | K1 |
| 2 | 2011 | Polyliner | P1 |
| 3 | 2012 | Kraft paper | K2 |
| 4 | 2012 | Polyliner | P2 |

Samples were compared in the following way: K1 with P1 and K2 with P2. Comparison of K1 and K2 or P1 and P2 would not be correct since tobacco quality depends on weather, soil and similar conditions and varies from year to year. That is why only tobacco samples from the same year were compared.

Organoleptic analysis was done checking the aroma, colour and its intensity, elasticity and oiliness of the tobacco. Tobacco aroma may be juicy, sweetish, harsh, fruity, etc. Tobacco colour may vary from lemon to brown; it can be uniform or not. Regarding oiliness and elasticity, tobacco may be oily, dry, crispy, soft, gummy, etc. L*a*b colour space is known for its uniform and precise determination of the colour. 'L' shows the luminance (lightness) of the material on the scale from 0 (black) to 100 (white); 'a' scale ranges from negative (green colour) to positive (red colour) values, and 'b' scale ranges from negative (blue) to positive (yellow) values (Fig. 3) (Sachidananda, 2008).

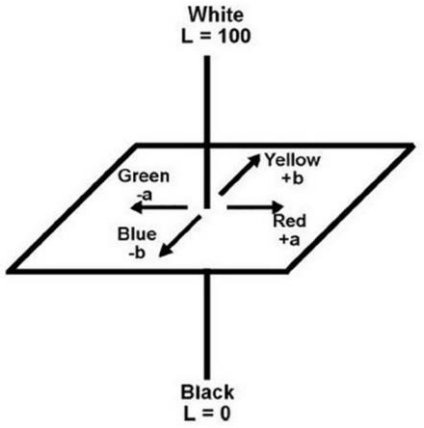


**Figure 3.** L*a*b colour space (Sachidananda, 2008).

For the analysis the following equipment and software were used: Standard fiber optic spectrometer Avantes AvaSpec-2048; AvaSoft, ver. 7.4; Microsoft Office Excel 2010; StatSoft STATISTICA, ver. 7.0. For spectrometric anlysis was used Avantes manual (Avantes, 2006).

L*a*b parameters were measured 10 times for each sample, since 'tobacco cake' has lighter and darker leaves. Prior every measuring session spectrometer was calibrated.

Gas Chromatography – Mass Spectrometry method (GC-MS) was used to identify the aromatic composition and volatile compounds of the tobacco samples. Analysis was performed with gas chromatograph GC-8000 from Fisons Instruments (Italy) with mass detector MD-800 and column Supercowax (fused silica capillary column 30 m length; 0.32 mm diameter; 65 μm film thickness; blue/plain hub). Helium was used as a carrier gas.

The beginning of the cycle started at 50 °C for 3 minutes and then the temperature would increase constantly by 3 °C per minute until reaching 250 °C. For identification of compounds, Library of chemical compounds and spectrums NIST (National Institute of Standards and Technology, USA) was used.

Determination of total alkaloids and reducing sugars in tobacco was done by colorimetric detection using the Skalar flow analyser. Moisture determination (oven volatiles) of tobacco products was done by oven drying. It was done according to Internal Standards of Philip Morris Q (KH) 0202 and Q (KH) 0210.

Alkaloids with a pyridine nucleus are determined by their reaction with cyanogen chloride in presence of sulfanilic acid. Formed imine derivative has a maximum absorption at 460 nm. Reducing sugars are determined by their reaction with p-hydroxybenzoic acid hydrazide in alkaline medium at 85 °C. Formed yellow osazone has a maximum absorption at 410 nm (DeBardeleben, 1987).

The analytical results (peak heights) are automatically processed by the system. The final results are expressed in % weight of dry tobacco.

## RESULTS AND DISCUSIONS

Despite the fact that the tobacco samples are of the closest equivalent grades, tobacco is a biological material and may be harvested during slightly different level of ripeness, which means that there will be no completely equal 'tobacco cakes'. Tobacco is very sensitive to internal and external conditions during its post-harvest operations.

In Tables 2 and 3 are listed aroma, colour, elasticity and oiliness of the samples.

**Table 2.** Organoleptic parameters of the samples from the year 2011

| Parameters | Sample | |
|---|---|---|
| | K1 | P1 |
| Aroma | Typical, deep, strong, sweetish | Typical, mild, sweetish |
| Colour | From orange to dark brown | From orange to brown |
| Elasticity, Oiliness | Dry, crispy | Less dry, soft |

**Table 3.** Organoleptic parameters of the samples from the year 2012

| Parameters | Sample | |
|---|---|---|
| | K2 | P2 |
| Aroma | Typical, mild, sweetish | Typical, mild, from sweetish to fruity |
| Colour | From orange to deep orange | From yellowish to orange |
| Elasticity, Oiliness | From oily to crispy, soft | Oily, soft |

Sample K1 has stronger aroma in comparison with sample P1. Also sample K1 is slightly darker. According to elasticity and oiliness characteristics, sample P1 is softer and less dry. Stronger aroma of the sample K1 is probably the result of more opened storage conditions inside of the C-48 carton with kraft paper liner. Air can go through the 'tobacco cake' accelerating the process of ageing. Also higher level of reducing sugars in the sample K1 can be the reason of such strong aroma. To ascertain this, the levels of total alkaloids and reducing sugar were measured. Colour is almost the same except for some areas of sample K1 where tobacco is dark brown. For more precise results of colour comparison, L*a*b analysis was performed. Sample P1 is less dry and softer in comparison with sample K1.

Aroma of the sample K2 is slightly deeper; however both samples have mild and sweetish aroma (the sample P2 had even fruity aroma) – see volatile compounds in Table 5. As was mentioned before, more opened storage conditions inside of C-48 carton with kraft paper liner may result in deeper aroma. Also the smaller difference in aroma between the samples from the year 2 in comparison with the samples from the year 1 may be the result of small difference in levels of reducing sugars influenced by one-year ageing. In general, the colour of both samples is quite similar. However, the sample K2 has deep orange leaves and P2 has yellowish leaves. For more precise results of colour comparison L*a*b analysis was performed. The sample P2 is oily and soft. The sample K2 is slightly crispier, however soft as well.

Since the colour of the samples from the same year looks very similar, L*a*b analysis was performed. Each parameter was measured 10 times. Average values of L*a*b analysis results are shown in Table 4.

**Table 4.** Average value of L*a*b analysis results

| Parameters | Sample | | | |
|---|---|---|---|---|
| | K1 | P1 | K2 | P2 |
| Average L | 22.435 | 33.075 | 32.923 | 46.428 |
| Average a | 9.92 | 12.446 | 12.612 | 11.159 |
| Average b | 26.021 | 24.369 | 28.123 | 31.496 |

The following figures (Figs 4, 5) show comparisons of 'L' parameter values between samples K and P from different years.

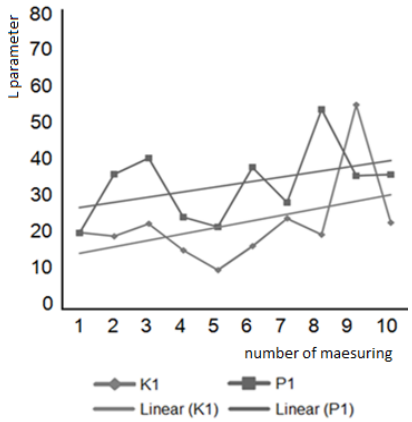


**Figure 4.** Luminance of the samples from the year 1.

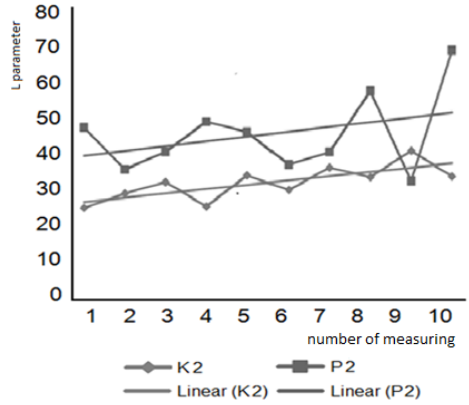


**Figure 5.** Luminance of the samples from the year 2.

The higher L values the lighter the material is. Both samples P1 and P2 have higher luminance comparing to the samples K1 and K2. Darker colour of the samples aged in C-48 cartons with kraft paper liner may be the result of faster ageing process.

L*a*b analysis results were processed in StatSoft STATISTICA software to compare the colour uniformity of the samples (Fig. 6).

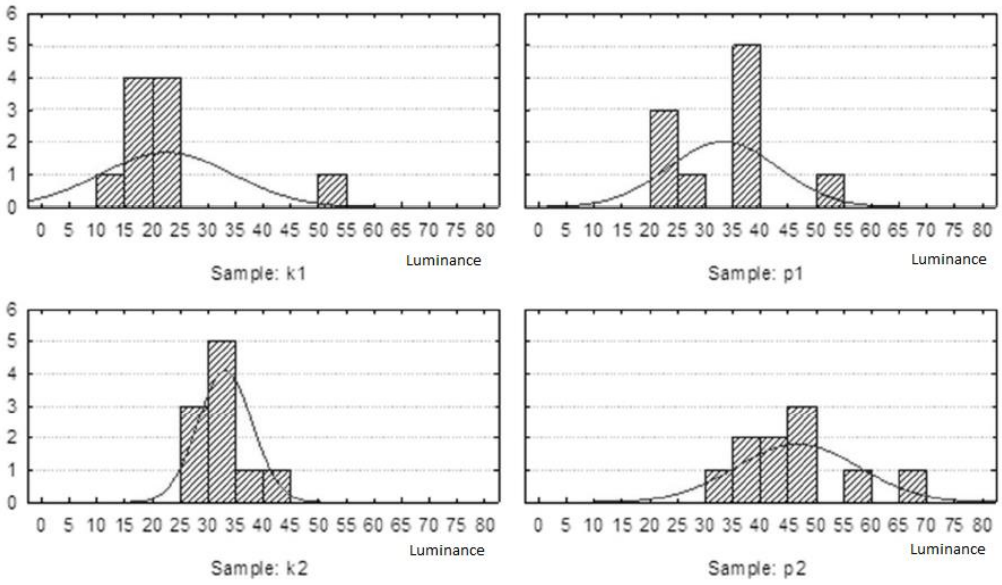


**Figure 6.** Colour uniformity of the samples.

The colour of the sample K2 is more uniform in comparison with the sample P2. Both samples K1 and P1 have low uniformity of colour. However, if we compare the changes between samples from different years aged in the same conditions, we can see that colour uniformity of tobacco aged in C-48 carton with kraft paper liner may change during the ageing process. But this information may vary since the sample size was 500 grams out of 200 kg 'tobacco cake'.

GC-MS analysis results are shown in the Fig. 7 and Fig. 8. Many volatile compounds were detected during this experiment. The list of compounds with highest peaks is shown in Table 5.

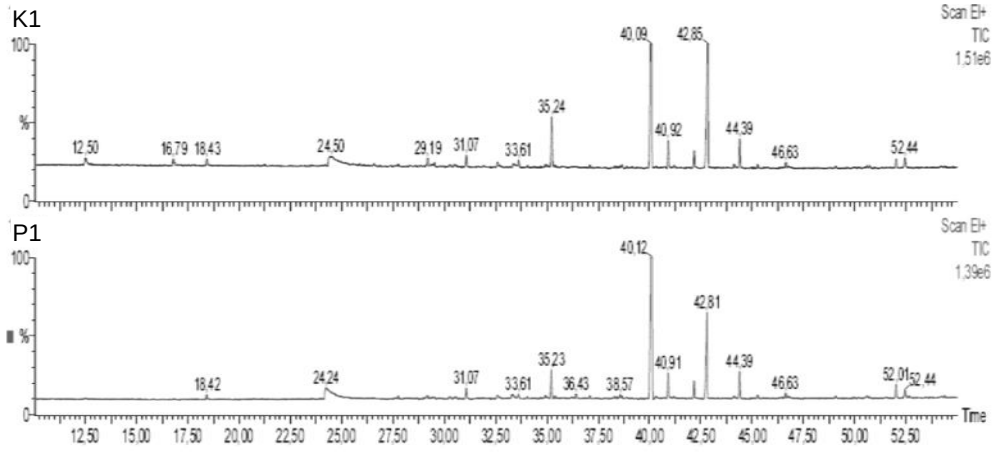


**Figure 7.** GC-MS analysis of volatile compounds of the samples from the year 2011 (K1, P1).

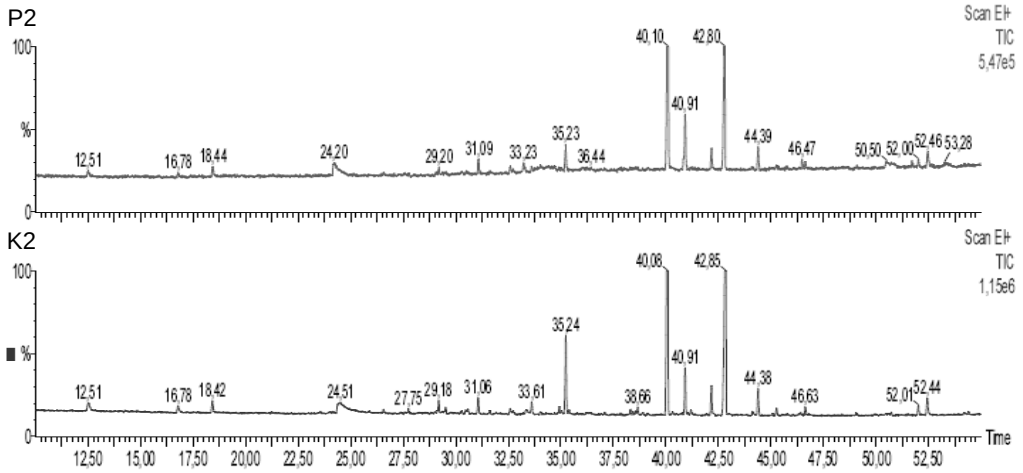


**Figure 8.** GC-MS analysis of volatile compounds of the samples from the year 2012 (P2, K2).

**Table 5.** Volatile compounds of the samples

| Peak, time (min) | Compound | Peak, time (min) | Compound |
|---|---|---|---|
| 12.5 | aliphatic | 40.92 | benzyl alcohol |
| 16.79 | aliphatic | 42.17 | 2-phenylethanol |
| 18.43 | aliphatic carbonyl | 42.85 | aliphatic alcohol |
| 24.50 | acetic acid | 44.39 | acetyl pyrrole |
| 29.19 | aliphatic carbonyl | 46.63 | aliphatic |
| 33.61 | 2H-pyran-2-on | 52.01 | aromatic aldehyde |
| 35.24 | *unidentified* | 52.44 | heterocyclic |
| 36.24 | sesquiterpenic carbonyl | 55.20 | aliphatic |
| 40.09 | nicotine | 56.54 | aliphatic alcohol |

The results of GC-MC analyses show quantitative and qualitative biochemical composition of the samples. The highest peaks on all chromatograms are the peaks of nicotine, the main alkaloid in the tobacco leaf. Second highest peaks are the peaks of aliphatic alcohol with retention time of 42.85 minutes. Also following peaks look sizeable: the peak of unidentified compound with retention time of 35.24 minutes and the peak of benzyl alcohol with retention time of 40.92 minutes. Comparing quality and quantity of the peaks, we can say that the type of liner does not affect biochemical composition of the tobacco leaf, which means that both types of packaging can be used without unwanted changes in the product.

Results of total alkaloids and reducing sugars levels were corrected according to percentage of oven volatiles. The amount of total alkaloids and reducing sugar on dry weight basis was determined after correction. Values for total alkaloids can be identified with the value for alkaloid nicotine content because other alkaloids are present in negligible amounts. Results of total alkaloids, reducing sugars and oven volatiles can be found in Table 6.

**Table 6.** Total alkaloids, reducing sugars and oven volatiles determination

| Parameters | Sample | | | |
|---|---|---|---|---|
| | K1 | P1 | K2 | P2 |
| TA (%) | 2.81 | 3.17 | 2.82 | 3.00 |
| RS (%) | 10.58 | 7.95 | 9.90 | 9.05 |
| OV (%) | 8.80 | 9.30 | 9.20 | 8.50 |

All of the samples have normal levels of total alkaloids and reducing sugars, according to standards for NLS FCV tobacco (1.5–3.5% of nicotine, 7–18% of reducing sugars).

Both samples aged with kraft paper liner have lower level of nicotine comparing to the samples aged with polyliner; however, the level of reducing sugars is higher. The amount of nicotine and that of sugars usually have an inverse relationship. As it was assumed during organoleptic analysis, the difference in reducing sugars levels between samples K2 and P2 is not that big whereas the level of reducing sugars in the sample K1 is much higher than in the sample P1 (it may be the reason of such difference in aroma between these two samples).

## CONCLUSION

The following conclusions based on the results can be done: tobacco aged with kraft paper liner has a stronger and deeper aroma in comparison with tobacco stored with polyliner. Tobacco aged for two years has a stronger aroma than tobacco aged for one year. Comparing the changes between samples from different years, aged in the same conditions, we can see that colour uniformity of tobacco aged in C-48 carton with kraft paper liner may change during the ageing process. But this parameter may vary.

Comparing the quality and quantity of the peaks on the chromatograms, we can say that the type of liner does not affect biochemical composition of the tobacco leaf. The highest peaks on all chromatograms are the peaks of nicotine, second highest peaks are the peaks of aliphatic alcohol, next peaks are unidentified compound and the benzyl alcohol. It means that both types of packing can be used without unwanted changes in the product. Ageing in a C-48 carton with kraft paper liner can proceed faster because there are more open storage conditions (air goes through 'tobacco cake' easier), whereas in a carton with polyliner, conditions are more stable and not affected by weather or season of the year.

In both types of storage levels of total alkaloids and reducing sugars stay in ranges of standards for NLS FCV tobacco For sample K1 is the measured value of total alkaloids 2.81%, for sample P1 3.17%, for sample K2 2.82% and for sample P2 3.00%. However, tobacco aged in C-48 cartons with kraft paper liner has a higher level of reducing sugar. For sample K1 is the value 10.58, for sample P1 7.95%, for sample K2 9.90% and for sample P2 9.05%. These values correspond to the values indicated by Manickavasagan, at al., 2007.

According to conclusions, the following can be recommended: for faster ageing in stable weather conditions kraft paper liner can be used. This type of storage can be recommended for domestic use of tobacco. Polyliner is better for maintaining stable conditions of the product during ageing as well as during the transportation.

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# Winter wheat grain baking quality depending on environmental conditions and fertilizer

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**Abstract.** Yield and quality of wheat grain depends on many factors such as environmental conditions, soil quality, genetic parameters and fertilization, especially nitrogen fertilizer which is one of the most important factor influencing quality parameters of winter wheat. Field experiments were done at the Research and Training Farm Vecauce of the Latvia University of Agriculture during 2013 till 2015. The aim of our study was to determine effect of nitrogen fertilization and environmental conditions on winter wheat (*Triticum aestivum* L.) variety 'Kranich' grain quality parameters. The investigated factors were six different nitrogen application norms (0 – control, 85, 153, 175+S21, 187 N kg ha$^{-1}$) and differential nitrogen norm according to chlorophylmeter (Konica Minolta Ltd.) data 180 N kg ha$^{-1}$ in 2013, 150 N kg ha$^{-1}$ in 2014 and 205 N kg ha$^{-1}$ in 2015. One more variant was added – 175 N kg ha$^{-1}$ in 2015. During the study years the meteorological conditions were significantly different. Our trials results showed that protein content suitable for bread making was obtained in variants N175+S21, 180 and 187 in year 2013, in all N application forms in 2014, but in 2015 – in all applications except N0, N175+S21, N85. The meteorological conditions had factor influence ($\eta^2$) 46% on protein content, but fertilizer application – 35%. Strong significant relationship at the 0.01 probability level between protein content and gluten content (r = 0.99), sedimentation value (r = 0.97) and falling number (r = 0.74) was found.

**Key words:** winter wheat, protein content, gluten content, fertilizers.

## INTRODUCTION

Wheat is an important cereal in Latvia national economy providing human population with bread. The best available technology should be used for fertiliser and pesticide handling and used in a way to provide the maximum effectiveness with the minimal negative side effect on crop and environment (Alam, 2014). Nitrogen application is one of the crucial factors for successful implementation of crop management practices used in wheat production system. Lack of nitrogen (N) in plants was related to grain filling, which required greater nitrogen amounts to be applied later. To maximize the use of fertilizer N by crops, N should ideally be applied as it is required by the crop plants. The proportion of applied nitrogen taken up by the crop is affected by many factors including crop species, climate and soil conditions. A simple and reliable method for field determination of wheat nitrogen status is using of

chlorophyllmeter. It can be used to make quick and easy measurements of leaf greenness, which is positively related to leaf chlorophyll content (Murdock et al., 2004 & Marsh, 2014). More and more farmers looking for simple method which to help determine the crop N status. In Latvia there is no investigations about chlorophyllmeter usage in winter wheat.

There is limited demand for wheat with a low (< 12.0%) protein content in Latvia. Values less than 12.0% demonstrate nitrogen insufficiency reaching yield potential (Mašauskiene et al., 2006). Improvements in nitrogen management are needed to produce superior quality grain and satisfy market demands for baking quality. The baking quality of winter wheat depends greatly on variety, soil fertility, climatic conditions, crop density and fertilization (Johansson, 2002; Otteson et al., 2008; Linina & Ruža, 2014). The temperature, especially during the grain filling period, is the most important weather parameter. Johansson & Svensson (1998) have shown that 34% of variation temperature influenced protein content. Dupont & Altenbach (2003) conclude that genetic and environmental factors affect wheat quality mainly through their effects on grain protein content and composition.

The aim of our study was to determine effect of nitrogen fertilization and environmental conditions on winter wheat (*Triticum aestivum* L.) variety 'Kranich' grain yield and baking quality parameters.

## MATERIALS AND METHODS

Field trials were carried out in the Research and Training Farm 'Vecauce' of the Latvia University of Agriculture during 2013 till 2015. The 2012/2013 and 2013/2014 experiments were carried out on the fields with winter oilseed rape as the previous crop, but in 2014/2015 – spring wheat. The soil at the site was loam, Endostagnic Phaeozem (Loamic) (WRB, 2014) with humus content 17–25 g $kg^{-1}$, soil pH KCl – 6.6–7.2, plant available potassium content average, phosphorus content – average to high, sulphur content – low. Winter wheat variety 'Kranich' was used. The variety is characterized by good winter hardiness, high yield potential and A Class food grain quality. It is one of the most suitable for growing in the Baltic conditions by using intensive technology. Sowing was done in optimal time for Latvia's conditions, and used seed rate was 450 viable seeds per $m^2$. At sowing depending on the year winter wheat was fertilized with compound fertilizer rate N – 15, P – 20, K – 62 kg $ha^{-1}$ in 2012, N – 18, P – 34, K – 75 kg $ha^{-1}$ in 2013 and N – 11, P – 21, K – 45 kg $ha^{-1}$ in 2014.

The investigated factors were six different nitrogen application norms (0 – control, 85, 153 (85+68), 175+S21 (85+60(S14)+30(S7)), 187 (85+68+34) N kg $ha^{-1}$) and differential nitrogen norm according to chlorophylmeter (Konica Minolta Ltd.) data 180 (85+50+45) N kg $ha^{-1}$ in 2013, 150 (85+50+15) N kg $ha^{-1}$ in 2014 and 205 (85+70+50) N kg $ha^{-1}$ in 2015 (further N-test). One more variant was added – 175 (85+60+30) N kg $ha^{-1}$ in 2015. The first dose of nitrogen was given in spring at the beginning of wheat regrowth, the second time at the stem elongation and the third time – at the beginning of heading. Treatments were arranged in four replicated randomized blocks.

Grain yield was determined at 100% purity and 14% humidity after the grains was dried. After harvesting, grain samples per each plot were taken according to standard LVS – 270. Grain protein content, gluten content, sedimentation value or Zeleny index and starch content were determined by grain analyzer Infratec 1241 (Foss Tecator AB,

Sweden). The Hagberg falling number was measured by the Hagberg – Perten method using a Perten Instruments (Sweden) 'Falling number 1500' was assessed to LVS EN ISO 3093.

The meteorological conditions were significantly different in all research years. In 2013 and 2015 wheat overwintered succesfully, but in 2014 January and February were not favourable for plant overwintering and some of winter wheat did not survive. In 2013 vegetation started very late – at the end of April. In 2014 and 2015 in April air temperature was higher by 2.7 °C and 1.9 °C than the average for many years (Fig. 1). In 2013 May air temperature was 3°C higher than long-term and precipitation three times more than norm. In 2013 and 2015 June meteorological condition were very dry: precipitation was lower than long–term mean by 22.6 and 21.6 mm accordingly. In 2014 and 2015 June air temperature was lower than norm and in 2013 and 2015 was recorded sufficient precipitation. Air temperature in grain filling period – July – was close to the long–term mean in 2013 and 2015, but 3 °C higher than norm in 2014. August weather was favorable for harvesting in all research years.

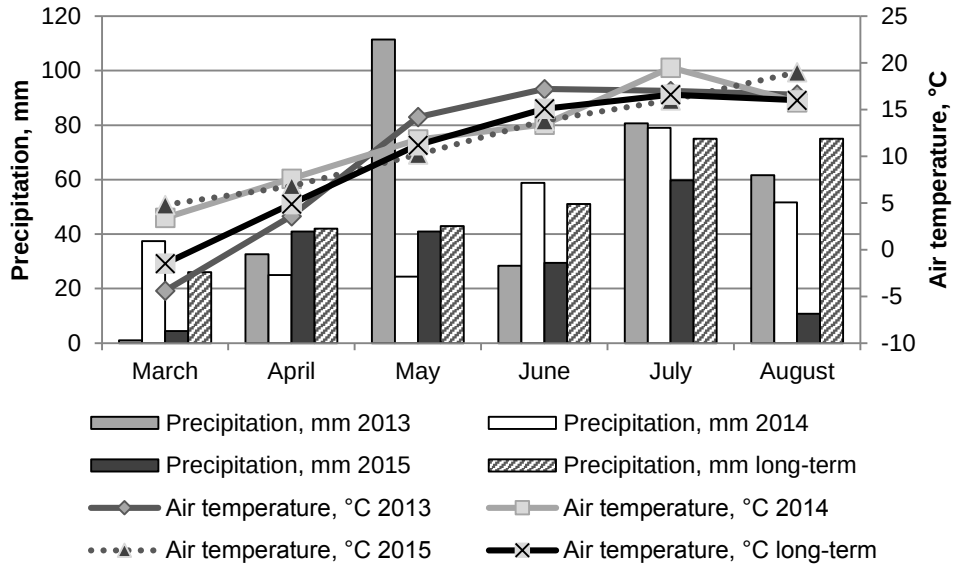


**Figure 1.** Meteorological conditions in 2013–2015 winter wheat vegetation period.

ANOVA procedures, the test of statistically significant differences at Fischer's criterion, impact factors influence ($\eta^2$) and correlation analyses were used for experimental data processing. The software MS Excel was used for data analyses.

## RESULTS AND DISCUSSION

Split topdressings of nitrogen fertilizer after spring green-up may improve nitrogen efficiency and increase yield and grain quality (Jarvan et al., 2012; Alam, 2014). In our research according chlorophyllmeter readings nitrogen norm for plant growth and development were differ year by year (180, 150 and 205 kg $ha^{-1}$ N) and provided baking

quality grain. Environmental conditions and plant supply with nutrients greatly influenced demand for nitrogen each year.

Wheat grains with protein content more than 12.0% are suitable for bread making. Our trials results showed that protein content suitable for bread was obtained in variants N175+S21, N-test and N187 ($LSD_{0.05}$ = 0.39) in year 2013, in all N application forms ($LSD_{0.05}$ = 0.29) in 2014, but in 2015 – in all N applications except N0, N175+S21, N85 ($LSD_{0.05}$ = 0.76) (Table 1). The baking quality parameters were influenced by environmental conditions (trial site, soil type, meteorological conditions) in trial years and nitrogen fertilizer. A significant impact ($P < 0.05$) of nitrogen application (35%) and meteorological conditions (46%) were noted on protein content. Similar results have been reported for winter wheat meteorological conditions significant impact to protein content (Cociu & Alionte, 2011; Linina & Ruža, 2014).

**Table 1.** Effect of nitrogen fertilizer on winter wheat grain quality parameters, 2013–2015

| Year | Nitrogen fertilizer rates | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | N0 | N85 | N153 | N175 | N175+S21 | N–test | N187 | $LSD_{0.05}$ |
| Protein content, % | | | | | | | | |
| 2013 | 9.10 | 9.70 | 11.40 | x | 12.50 | 12.00 | 12.60 | 0.39 |
| 2014 | 12.43 | 12.50 | 14.50 | x | 15.23 | 15.38 | 15.85 | 0.29 |
| 2015 | 9.70 | 9.97 | 12.55 | 12.92 | 10.80 | 14.02 | 13.62 | 0.76 |
| Average | 10.41 | 10.72 | 12.82 | 12.92 | 12.84 | 13.80 | 14.02 | x |
| Gluten content, % | | | | | | | | |
| 2013 | 16.08 | 17.65 | 22.65 | x | 25.65 | 24.43 | 25.98 | 1.29 |
| 2014 | 25.20 | 25.65 | 30.60 | x | 32.55 | 33.15 | 34.68 | 0.89 |
| 2015 | 18.20 | 19.00 | 26.35 | 27.77 | 21.60 | 30.77 | 29.40 | 2.28 |
| Average | 19.83 | 20.77 | 26.53 | 27.77 | 26.60 | 29.45 | 30.02 | x |
| Sedimentation value, mL | | | | | | | | |
| 2013 | 20.33 | 23.83 | 35.50 | x | 41.90 | 39.73 | 42.78 | 2.92 |
| 2014 | 35.43 | 39.63 | 58.10 | x | 62.65 | 62.98 | 63.38 | 3.94 |
| 2015 | 23.57 | 25.67 | 46.80 | 51.67 | 32.27 | 61.27 | 59.02 | 7.04 |
| Average | 26.44 | 29.71 | 46.80 | 51.67 | 45.61 | 54.66 | 55.06 | x |
| Falling number, s | | | | | | | | |
| 2013 | 371 | 341 | 379 | x | 364 | 365 | 370 | 19.36 |
| 2014 | 354 | 371 | 383 | x | 385 | 381 | 391 | 9.77 |
| 2015 | 334 | 343 | 369 | 359 | 375 | 388 | 379 | 14.41 |
| Average | 353 | 352 | 377 | 359 | 374 | 378 | 380 | x |

The use of nitrogen fertilizer increased gluten content in all trial years. The highest gluten content (34.68%) was obtained in year 2014 by using nitrogen fertilizer norm 187 kg $ha^{-1}$, also all N norms provided suitable gluten content for baking quality grains (25.20– 4.68%). Meteorological conditions (40%) and nitrogen fertilizer (51%) showed significant impact ($P < 0.05$) on this parameter.

Sedimentation value or Zeleny index is the most important protein quality character trait. In all trial years sedimentation value increased with increasing nitrogen fertilizer norm. Nitrogen fertilizer influence on sedimentation value was found also in other scientist trials (Mašauskiene et al., 2006; Linina et al., 2012). Three year average sedimentation value above nitrogen fertilizer norm 153 kg $ha^{-1}$ obtained second class quality (45.61–55.06 mL). These grains were useful for direct baking or mixing up with

comparatively weak flour. Meteorological conditions (30%) and nitrogen fertilizer norm (57%) showed significant influence ($P < 0.05$) on sedimentation value.

An important parameter of wheat grain quality is Hagberg falling number, which shows α-amylase activity and measure how far the break-down of starch has progressed in the kernel through enzymatic activities. Optimal falling number for good bread preparing according grain processor Dobeles dzirnavnieks (Dobele Miller) requirements is more than 230 s (Elite and A class > 280 s, I group > 270, II group > 250, III group > 230) (Dobeles Dzirnavnieks, 2015). Falling number higher than 280 s (334–391 s) was obtained in all trial years and standard – Elite was reached. High falling number indicated low α-amilase activity (Lunn et al., 2001) and is affected by the precipitation during grain maturation (Johansson, 2002). Our results showed significant impact ($P < 0.05$) of nitrogen fertilizer (43%) and meteorological conditions (11%) on falling number. In all investigated years precipitation at grain harvesting period were lower than long-term mean (Fig.1) and provided high falling number – to comply with bread baking quality requirements put forward by processors.

Different grain yields were obtained depending on trial year meteorological conditions and nitrogen application norms. Grain yield in 2013 varied from 4.07–7.84 t $ha^{-1}$, in 2014 – 2.79–5.20 t $ha^{-1}$, and in 2015– 4.23–10.20 t $ha^{-1}$. The highest winter wheat yield (10.20 t $ha^{-1}$) was obtain in 2015 with N application N175+S21, but – with low protein content (10.80%), it could be explained by lack of nitrogen for obtaining food grain quality. This is in accordance with results of other studies (Jarvan et al., 2012) where with increasing yields the protein and wet gluten concentrations in grain decreased. Correlation between grain yield and quality parameters was not found. Results connected with Linina & Ruza (2014) and Kaya & Akcura (2014) reports. Correlation analyses of qualitative parameters of wheats shows a strong significant relationship at the 0.01 probability level between protein content and gluten content r = 0.99 (Table 2), sedimentation value r = 0.97 and falling number = 0.74 ($\alpha_{0.01} = 0.60$, $n = 19$). Similar results were found by Kaya & Akcura, 2014 and Cioineag & Cristea, 2015. Close significant negative correlation at the 0.01 probability level was found between protein content and starch content (r = -0.98). Close significant correlation was found also between gluten content and sedimentation value, and falling number.

**Table 2.** Correlation between winter wheat grain quality parameters

| Parameter | Grain yield | Protein content | Gluten content | Sedimentation value | Falling number |
|---|---|---|---|---|---|
| Protein content | -0.11 | | | | |
| Gluten content | -0.06 | 0.99 | 1.00 | x | x |
| Sedimentation value | 0.02 | 0.97 | 0.98 | 1.00 | x |
| Falling number | 0.06 | 0.74 | 0.74 | 0.75 | 1.00 |
| Starch content | 0.02 | -0.98 | -0.98 | -0.98 | -0.77 |

Probability level $\alpha_{0.01} = 0.60$, $n = 19$.

Similar researches in future have to be done in winter wheat for high grain yield and baking quality obtaining under different nitrogen management strategies. The usage of chlorophyllmeter is increasing in farms that are why more research has to be done in future.

## CONCLUSIONS

The baking quality parameters (protein content, gluten content, sedimentation value and falling number) were significantly ($P < 0.05$) influenced by environmental conditions (trial site, soil type, meteorological conditions) in trial years and application of nitrogen fertilizer. Protein content suitable for bread was obtained in variants with nitrogen fertilizer more than 153 kg $ha^{-1}$, except in year 2013 – N153 kg $ha^{-1}$ and 2015 – N175+S21 kg $ha^{-1}$. Nitrogen fertilizer increased gluten content and sedimentation value in all trial years. Correlation between grain yield and quality parameters was not found. Strong significant relationship at the 0.01 probability level between protein content and gluten content, sedimentation value and falling number was found. Further research on N fertilizer usage efficiency in winter wheat is needed.

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# Physical and Chemical Properties of Extruded Pea Product

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**Abstract.** Peas (*Pisum sativum* L.) are a good source of protein, dietary fibre, and certain minerals, thus making them valuable nutrients in human diet. Unfortunately, peas are not commonly used in human diet due to their long cooking time. New products should be manufactured to increase the presence of peas in human diet. In order to make the grey peas easier for people to consume, extrusion cooking was used. Due to varying recipes, different products were obtained. Peas of the variety 'Bruno' with and without the addition of wheat and oat flour and egg powder were used in the experiments. Protein, fat and starch content of these products was analysed chemically but their pH, size, hardness, and volume mass was measured using physical methods. The average pH for all the samples was 7.3 ± 0.5, size differences ranged from 5.4 ± 0.4 mm to 10.3 ± 0.5 mm in length and 6.4 ± 0.2 to 11.7 ± 0.8 mm in width. More fat was found in the sample with onion flavour – up to 9.5 ± 0.5 g 100 $g^{-1}$ – but the least amount of fat was found in the sample without any seasoning – 0.6 ± 0.05 g 100 $g^{-1}$ on average. The average starch content was 23 ± 2 g 100 $g^{-1}$, while the highest protein content was discovered in the sample where grains and egg powder had not been added – 26.9 ± 0.2 g 100 $g^{-1}$, and the lowest – 18.6 ± 0.5g 100 $g^{-1}$ – in the sample with the largest grain proportion. The samples with the highest volume mass were the ones with added egg powder – 43 ± 2 N and 387 ± 2 g $L^{-1}$. The obtained results show that the largest and crispiest sample was acquired using only pea flour, and pea and wheat flour mixed in the proportion 1:1.

**Key words:** Peas, extrusion-cooking, characteristics.

## INTRODUCTION

As peas contain substantial levels of protein, their potential nutritional value is high. Peas also contain rather high levels of starch, although it varies from 33 g 100 $g^{-1}$ to 48 g 100 $g^{-1}$ in dry matter. For winter and spring cultivars the difference is usually less considerable, for example, in winter cultivars of the white pea the reported starch content is 47.5 g 100$g^{-1}$ for dry matter but for spring cultivars it is 50.0 g 100 $g^{-1}$ for dry matter (Gatel & Grosjean, 1990). Potentially, peas are a very valuable foodstuff in terms of their energy-yielding potential. X-ray diffraction studies (French, 1984) show there are crystalline regions within the starch granule that are more resistant to acidic and enzymatic hydrolysis, leading to reduced starch digestibility in raw peas. Trypsin inhibitor activity levels may be reduced by heat processing, and the susceptibility of starch to enzymes can be increased by gelatinisation, or any other process that destroys the granular structure of starch (Holm et al., 1985); heating may also lead to the loss of α-amylase inhibitors (Alonso et al., 2000; Al-Marzooqi & Wiseman, 2009).

Extrusion technology is well-known in the plastics industry but it has also become a widely-used technology in agri-food production where it is referred to as extrusion-cooking. It has been employed for the production of so-called engineered food and special feed. The extrusion-cooking of raw vegetable materials means the extrusion of ground material at baro-thermal conditions. With the help of shear energy exerted by the rotating screw, and the additional heating of the barrel, the foodstuff is heated to its melting or plasticating point (Van Zuilichem, 1992; Moscicki, 2011).

In this changed rheological status the food is conveyed through a die under high pressure. Then the product expands to its final shape. As a result, the physical and chemical properties of the extrudates are very different compared to those of the raw materials. Extrusion-cookers are high-temperature short-time equipment capable of performing cooking tasks under high pressure. This is advantageous for vulnerable food and feed, as exposure to high temperatures for a short time restricts unwanted denaturation effects on, for example, proteins, amino acids, vitamins, starches, and enzymes (Moscicki, 2011).

Unfortunately, peas are not commonly used in human diet due to their extended cooking time. The aim of the study was to develop new products to increase the presence of peas in human diet, and their chemical and physical properties were studied and described.

## MATERIALS AND METHODS

In order to create new grey pea products that would be easier to consume, extrusion-cooking was used for treatment. Various recipes were used to acquire different products. Peas of the variety 'Bruno' with and without the addition of wheat and oat flour and egg powder were used in the experiments.

Peas of the variety 'Bruno' from the State Priekuli Plant Breeding Institute were used in the experiments, as well as oat and wheat flour. The extrusion-cooking was carried out at Milzu Ltd. using a twin-screw extruder. Cinnamon, walnut, sugar, cocoa and baking powder were purchased from Gemoss Ltd.

Physical and chemical analyses were carried out at the Latvia University of Agriculture. Protein content (LVS EN ISO 5983-2:2009), volume mass (gravimetrical), size (measurement), pH (ГОСТ 26180-84, met.), fat content (ISO 6492:1999), starch content (LVS EN ISO 10520), hardness (Texture Analyzer, TA.XT.plus, pre-test speed 1.5 mm $sec^{-1}$; test speed 1 mm $sec^{-1}$; post-test speed 10 mm $sec^{-1}$; difference 5 mm; die: P/2 DIA) were measured and analysed. The recipes and abbreviations for the base samples are shown in Table 1.

As in pre-experiments the obtained samples were rather small and hard, so different additives were used to increase product size, aeration, thus lowering volume mass and hardness. Egg powder was added to increase the amino acid content of pea products. Other parameters such as pH were used to ascertain whether there are differences in the products that could be caused by the ingredients and treatment.

**Table 1.** Used ingredients and abbreviations for samples

| Sample No. | Used ingredients (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L |
| 1 | 91 | 9 | | | | | | | | | | |
| 2 | 89 | 9 | | 1 | | | | | | | | |
| 3 | 78 | 9 | 1 | 1 | | 7 | 5 | | | | | |
| 4 | 72 | 9 | 0.2 | 1 | 4 | 9 | 4 | | | | | |
| 5 | 69 | 9 | 0.2 | 1 | 4 | 9 | 4 | | 4 | | | |
| 6 | 66 | 8 | 0.3 | 1 | 4 | 8 | 4 | | 4 | | 4 | 0.1 |
| 7 | 66 | 8 | 0.2 | 1 | 4 | 8 | 4 | | 4 | 4 | | |
| 8 | 39 | 5 | 0.2 | | | | | 39 | 8 | | 8 | 0.2 |
| 9 | 44 | 6 | 0.2 | | | 44 | | | 6 | | | |
| 10 | 71 | 8 | 0.2 | | | 8 | 4 | | 4 | 4 | | |

A – Grey peas; B – Water; C – Salt; D – Baking powder; E – Egg powder; F – Wheat; G – Oat; H – Maize; I – Sugar; J – Cocoa; K – Walnut; L – Cinnamon.

In addition, salty and sweet seasonings were added to enrich the products. The abbreviations for the seasoned samples are shown in Table 2.

**Table 2.** Abbreviations for seasoned samples

| Base sample | Grill | Onion | Almond | Chocolate |
|---|---|---|---|---|
| 1 | 1-G | 1-O | - | - |
| 2 | 2-G | 2-O | - | - |
| 3 | 3-G | 3-O | - | - |
| 4 | 4-G | 4-O | - | - |
| 5 | - | - | 5-A | - |
| 7 | - | - | - | 7-C |
| 9 | - | - | 9-A | - |
| 10 | - | - | - | 10-C |

Grill and fried onion spices from Bairons LBC were used to make salty products but cocoa and almond flour from Gemoss Ltd. were used to make sweet products.

The mathematical analyses of the data was conducted using ANOVA in MS Excel, and all the chemical parameters were calculated on the basis of dry matter. 95% was used as the level of significance.

## RESULTS AND DISCUSSION

The volume mass of peas can be reduced from 667 ± 1 g $L^{-1}$ in non-extruded peas to 127 ± 1 g $L^{-1}$ (Fig. 1) in the extrudes of peas, and in a mixture composed of 50% peas and 50% of wheat flour. No significant differences were observed in the samples in which only pea flour was used compared to the samples with added baking powder, which is used in the food industry to achieve better aeration.

Depending on the additives used in the extrusion mix, samples with different volume mass were obtained. The heaviest samples were the ones with added walnut and egg powder, as these hardened the mixture. The samples where oat flour was added had a slightly bigger volume mass but no significant differences were discovered. That was also true when examining seasoned samples and their sample bases, as the coating layer was thin.

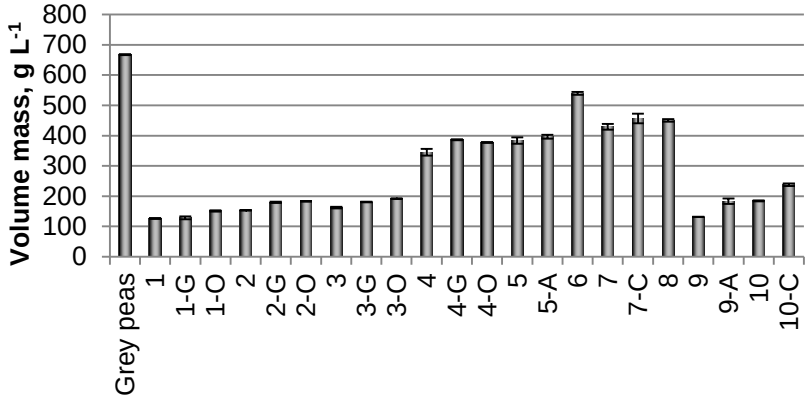


**Figure 1.** Volume mass of extruded pea (*Pisum sativum* L.) samples.

The samples to which egg powder had been added before extrusion were of significantly smaller size (Fig. 2) compared to those that did not have this additive. However, the samples with wheat and oat flour added to the extrusion mix were not symmetrical in size, as their length and width differed up to 5.6 mm from the samples extruded from pea flour only. The last ones were of similar size to those that had more wheat flour (44%) added to the extrusion mix.

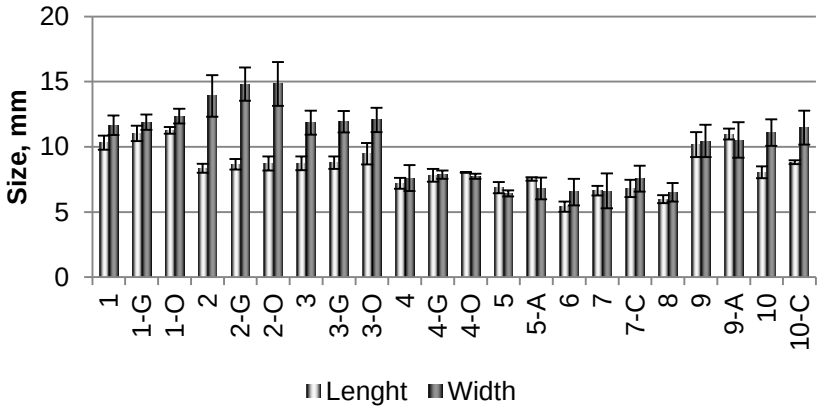


**Figure 2.** Sizes of extruded pea (*Pisum sativum* L.) samples.

The pellet length of samples without egg powder was 10.3 ± 0.5 mm and width 11.7 ± 0.7 mm but the length of samples with added egg powder was 7.2 ± 0.4 mm and width 7 ± 1 mm.

Significant differences were observed in comparing the size of different samples. For the length $\alpha = 0.05$, $p = 2.5 \cdot 10^{-75}$ but for the width $\alpha = 0.05$, $p = 6 \cdot 10^{-111}$; between samples $\alpha = 0.05$, $p = 3 \cdot 10^{-128}$. However, no significant differences were discovered in comparing base samples and coated ones.

All the coated samples had lower pH compared to the base samples they were made of. The biggest differences were observed in samples where egg powder was used in the extrusion mix. In the samples with grill coating pH was 0.9 units lower but in the samples with onion coating it was 0.7 units lower. Other sample differences did not exceed 0.6 units. Even though there are mathematical differences ($p = 3 \cdot 10^{-26}$ $\alpha = 0.05$), they are most likely caused by the standard error that did not exceed 0.1.

The pH for extruded pea (*Pisum sativum* L.) samples ranged from 6.27 to 7.84. The highest pH was observed in the sample with added egg powder but in the other extrudates pH was lower than 6 (Fig. 3). All the samples were pH neutral and no samples had pH lower than 5.

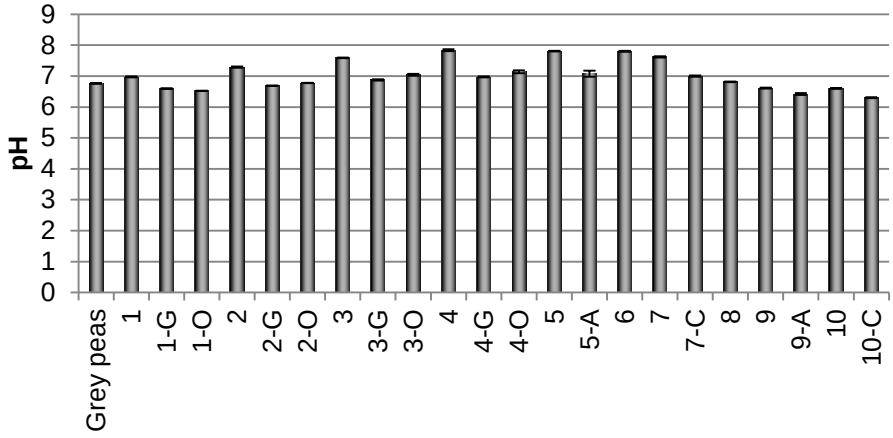


**Figure 3.** pH of extruded pea (*Pisum sativum* L.) samples.

The hardness of different samples was observed to range from 7.0 ± 1.7 N to 58.9 ± 10.1 N (Fig. 4.). The lowest level of hardness was recorded in the samples that were extruded from pea flour only, without any additional flour and other additives, the hardness being 7.9±0.8 N, and in samples with 44% added wheat flour—7.0 ± 1.7 N.

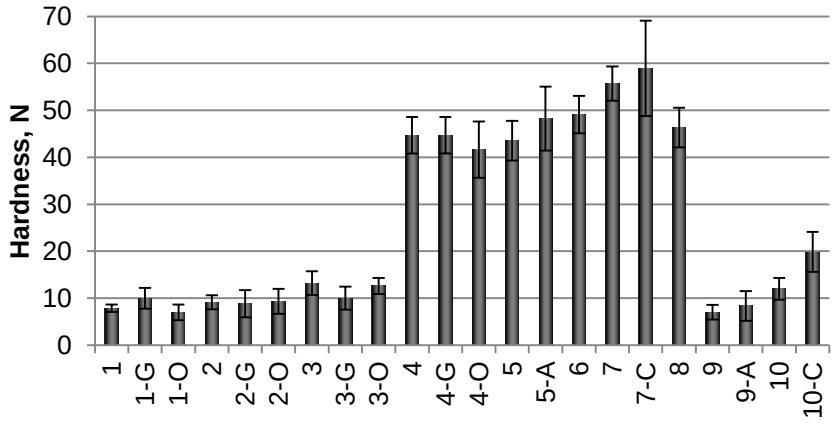


**Figure 4.** Hardness of extruded pea (*Pisum sativum* L.) samples.

The hardness of samples with added baking powder was 9.1 ± 1.5 N. The hardest samples were the ones with added egg powder, the hardness being 55.7 ± 3.6 N. Significant differences $\alpha = 0.05$; $p = 7.01 \cdot 10^{-9}$ were observed in comparing the samples. Only the sample with chocolate coating was harder by 7.9 N compared to its base, still no significant differences were observed between them.

No significant differences were found in the comparison of non-extruded pea and extruded pea protein content but maize flour decreased the protein content in the samples by more than 30%, as maize had only 8.1 ± 0.2 g 100 $g^{-1}$ protein in dry matter (Fig. 5).

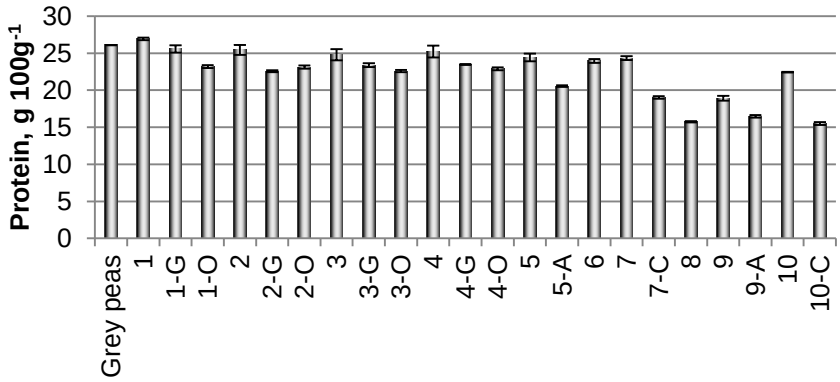


**Figure 5.** Crude protein content in extruded peas (*Pisum sativum* L.)

The samples with added egg powder had 25.2 ± 0.7 g 100 $g^{-1}$ protein in dry matter, while the sample made from pea flour had 26.9 ± 0.2 g 100 $g^{-1}$ crude protein in dry matter. The average protein content for base samples was 24.7 g 100 $g^{-1}$ in dry matter. Lower protein content was observed in the coated samples. Overall, in the samples with grill seasoning the protein content was higher than in those with onion coating. Only the sample with added baking powder and onion coating had slightly larger protein content than the one with grill flavour. The biggest differences were found in the samples with chocolate coating – the protein content had decreased to 15.5 ± 0.5 g 100$g^{-1}$, whereas it had originally been 22.5 ± 0.2 g 100 $g^{-1}$.

However, the fat content in the extruded peas without coating reduced in the extrusion process from 1.06 ± 0.02 g 100 $g^{-1}$ to 0.6 ± 0.1 g 100 $g^{-1}$ (Fig. 6). The samples with added walnuts had the highest fat content – 5.15 ± 0.01 g 100 $g^{-1}$. The samples with added egg powder had a fat content greater than 1 g 100 $g^{-1}$. In the samples that had no fat-containing additives the fat content was lower than 1 g 100 $g^{-1}$.

As oil and syrup were used for coating the samples, the results show that, as expected, the total fat content in the coated samples is higher than in the ones without coating. There was an up to 9.5 ± 0.5 g 100 $g^{-1}$ additional fat content in the samples with onion coating. The lowest fat content observed in the coated samples was in the chocolate sample without egg powder – 2.1 ± 0.2 g 100 $g^{-1}$ fat in dry matter.

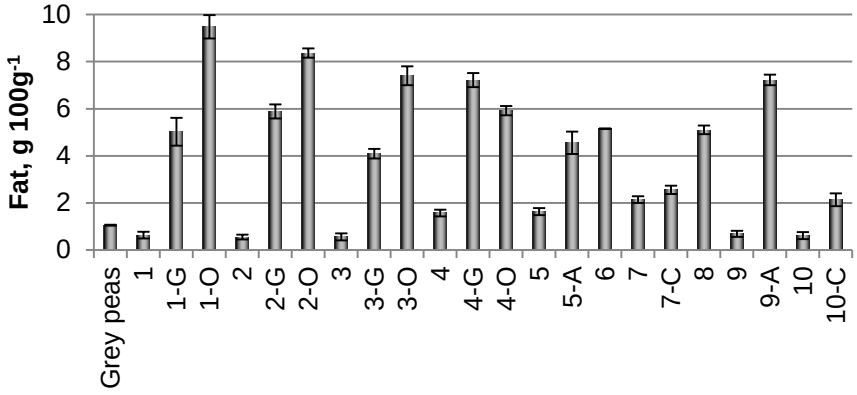


**Figure 6.** Fat content of extruded pea (*Pisum sativum* L.) samples.

The starch content in the extruded peas ranged from $20.5 \pm 0.5$ g 100 $g^{-1}$ to $26.1 \pm 0.7$ g 100$g^{-1}$ (Fig. 7). The highest starch content was found in the samples with added wheat.

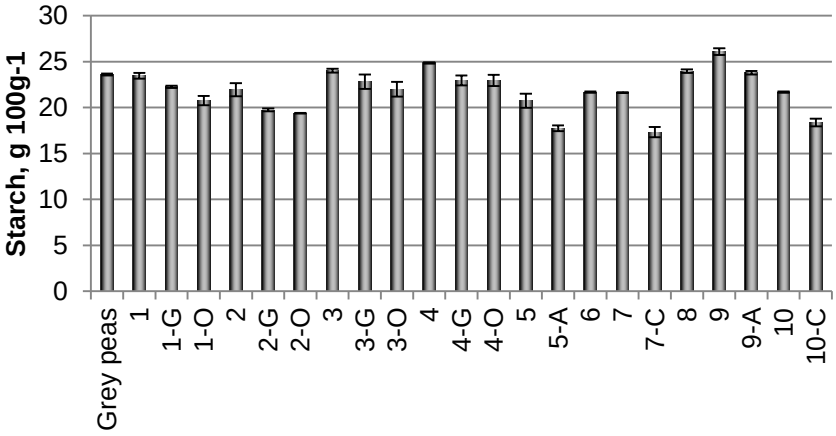


**Figure 7.** Starch content of extruded pea (*Pisum sativum* L.) samples.

Significant differences $\alpha$=0.05; $p$=1.05–$10^{-7}$ were observed in comparing the samples. As expected, the starch content decreased in the coated samples, especially in the sweet samples, as they had more coating than salty ones.

## CONCLUSIONS

If the amount of pea flour in the extruded products exceeds 60%, the protein content does not differ significantly from that of non-extruded peas. The coating decreases the protein and starch content in the samples while it increases the total fat content.

The volume mass of peas can be reduced to $127 \pm 1$ g $L^{-1}$ but significantly smaller samples are obtained with the use of egg powder as well as walnuts. For samples without

egg powder, the length was 10.3 ± 0.5 mm and the width was 11.7 ± 0.7 mm but for samples with added egg powder the length was 7.2 ± 0.4 mm and the width was 7 ± 1 mm.

Accordingly, samples made only of peas were 7.9±0.8 N hard, but samples with added egg powder were the hardest – 55.7 ± 3.6 N.

The starch content in the extruded peas ranged from 20.5 ± 0.5 g 100 $g^{-1}$ to 26.1 ± 0.7 g 100$g^{-1}$. The samples with added egg powder had 25.2 ± 0.7 g 100$g^{-1}$ protein in dry matter, while the sample made of pea flour had 26.9 ± 0.2 g 100 $g^{-1}$ crude protein in dry matter.

The obtained physical characteristics suggest that the best sample was obtained using only pea flour, and pea and wheat flour mixture in the proportion 1:1, whereas adding baking powder did not increase the aeration. No significant increase in protein content was observed with egg powder.

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# Developing a household vacuum cooking equipment, testing its performance on strawberry jam production and its comparison with atmospheric cooking

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**Abstract.** In this research, the performance of a kitchen appliance cooking equipment prototype, which can operate either under vacuum or at atmospheric pressure, is aimed to be developed and tested on strawberry jam production. Vacuum cooking applications were carried out at two different conditions as 17.5 and 25 minutes at 75 °C. Strawberry jam was also cooked at atmospheric pressure for 5, 10, 15 and 20 min. The effect of cooking conditions under vacuum and atmospheric pressure was determined by the following analysis; brix, color (L*, a*, b*) values, chroma (C*), hue (h°), pH value, titratable acidity, reducing and total sugar content (%), hydroxymethylfurfural content (HMF) and sensorial analysis. When the strawberry jam that is produced at atmospheric pressure is compared to the ones that are produced under vacuum, atmospheric cooked jam got higher Brix and was more viscous depending on the applied elevated temperature. HMF content of jam produced at atmospheric pressure was also found to be excessively high compared to the jam produced under vacuum. As it has been foreseen in the beginning of the study, vacuum cooking has been effective in reducing the HMF content of the strawberry jam due to the low temperature application. Sensorial quality of the vacuum-processed strawberry jam was superior in terms of color, appearance, consistency, taste and overall acceptance comparing to the atmosphere processed jams. This data could be utilized to contribute to the development of a household vacuum cooking equipment and the opportunity to produce with less harmful ingredients in home environment.

**Key words:** Jam, Strawberry jam, Vacuum Cooking, Evaporation, HMF.

## INTRODUCTION

Vegetables and fruits play an important role in our daily diet on account of their rich contents of nutritional compounds (Özel, 2006). Due to the high water contents of fruits and vegetables, their shelf-life is limited by microbial activity (Şahin et al., 1994). One of a/the most famous preserving methods in order to extend the shelf-lives of fruits and vegetables and to obtain various delightful products is called 'jam processing' (Kansci et al., 2003). Jam products are also popular food products due to their low cost,

their accessibility all the year around and their organoleptic features (Gałkowska et al., 2010).

Nowadays, besides using modern technologies in jam production at commercial levels, it is still being produced at the household levels (Gałkowska et al., 2010). In home-made jam production, particular proportions of the fruit and sugar are mixed. The fruit and sugar mix is then cooked to obtain a delightful blend which achieves sufficient storage capabilities. In order to acquire the desired final total soluble solid content, the mix is concentrated under excessive thermal treatment (Igual et al., 2013). This process leads to unsavoury colour, flavour and nutritional values of the output. These problems are due to the extreme temperature applications and long processing time (Garcia–Martinez et al., 2002). Also it leads to the formation of undesirable components such as hydroxymethylfurfural (HMF), furfural and melanoidins. Furthermore, producing jam at home is mostly insufficient for achieving the desired consistency. In The Aegean Region of Turkey, it has been observed that jam was kept under sunlight for a while after the heat treatment to thicken the jam's consistency as a traditional homemade method production. All these processes take too long to produce jam at home as well as its being difficult and tiring. On the other hand, keeping the jam under sun is not a hygienic method for food safety and could cause microbial load in the product.

In recent years, vacuum cooking has got remarkable development as an alternative cooking method because of the low-temperature application, the short processing time, the oxygen–reduced cooking environment, the better protection of nutritional value and the physical structure of the food (Garcia-Segoiva et al., 2007). Due to the low temperature and the oxygen content, vacuum cooking helps to protect the natural color and the flavour of the food (Andres-Bello et al., 2009). Whereas the reduction of the nutritional value and the formation of carcinogenic substances in food which is caused by high temperature is minimized, oxidation of the food could also be restricted by reducing the oxygen content of cooking medium with low temperature applications under vacuum. Low oxygen environment in the vacuum cooking process may reduce the formation of enzymatic and non-enzymatic aerobic spoilage reaction which occur during the traditional cooking process and affect the quality of the last product (Martinez-Hendez et al., 2013).

In the food industry, vacuum cooking method is generally preferred to produce jam, tomato paste and similar food products and the process is carried out in the closed vessels on industrial scale. However household cooking appliance that has a function capable of performing under vacuum is not available on the market. The aim of this study was to develop a kitchen appliance cooking equipment which can operate either under vacuum or at atmospheric pressure and to test its performance on strawberry jam production.

## MATERIALS AND METHODS

### Materials

Strawberries (*Fragaria x ananassa Duch.*) and sugar were purchased from a local market in İzmir. After strawberries were washed, stems and deteriorated parts were separated. The cleaned strawberries (moisture content, 90%) were weighed 600 g and sugar, which is commercial granulated sugar, was added 400 g on strawberries. Thus, osmotic dehydration and sugar penetration of strawberries were ensured like conventional domestic strawberry jam preparation in which strawberries are put in sugar

for an overnight before cooking. In addition, citric acid was added on strawberries and sugar mix just before the cooking process, to ensure the specific acidic flavor and decrease pH in order to provide the gel formation. The citric acid was powder and it was obtained from a local market and stored in dark and refrigerator condition (4 °C)

**Design of the vacuum cooking equipment prototype**

A kitchen appliance cooking equipment prototype was developed for aiming to work in a wide range of vacuum and precise temperature control as shown in Fig. 1.

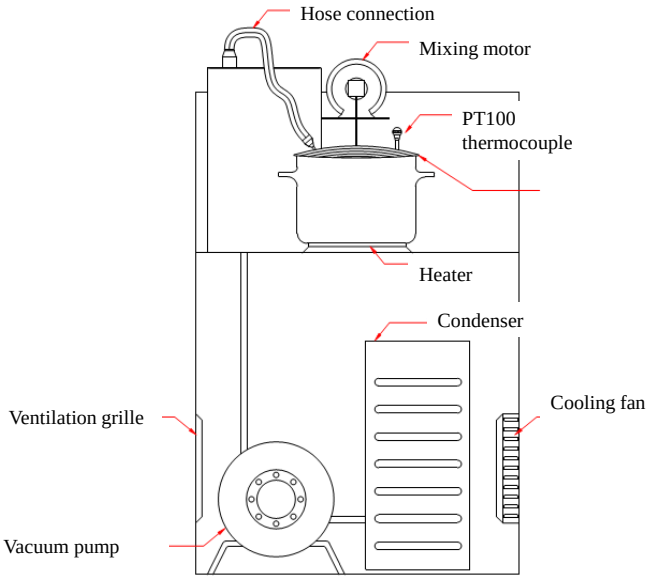


**Figure 1.** Developed vacuum cooking equipment prototype.

The Vacuum cooking vessel with capacity of 6 L includes a reductor mixer which can operate continuously or batch in the 0–50 Hz range. Electrical heater was used for heating about 1.5 kW also a temperature (PT 100) probe was adapted to system to measure the inside temperature of the vessel. The oily type of pump 0.55 Hp has been preferred to work in a wide range of vacuum. Applied vacuum level was measured inside the vacuum cooking vessel. Between the vessel and the pump, a condenser is needed to collect the water vapor coming from the jam while cooking process. The condenser is 1 kW and includes the refrigerant (R–404a).

Mixer rate, vacuum level and cooking time are controlled by PLC system. Electrical heater is also programed with PID control system. The inside temperature of vacuum cooking vessel, vapor temperature at condenser exit and pressure of vacuum cooking vessel are recorded per each 3 seconds.

**Strawberry Jam Production**

In this research, the strawberry jam was produced either under vacuum or at atmospheric pressure. Before production of strawberry jam, frozen strawberry and sugar mix (~1,000 g) was thawed in refrigerator (4 °C) during a day. Citric acid (1 g) was added on thawed strawberries and sugar mix. For the vacuum treatment the strawberry

jam was cooked at 75 °C and for 17.5 and 25 min under vacuum according to preliminary trial and the data obtained from literature. For the atmospheric treatment, the strawberry jam was cooked for 5, 10, 15 and 20 min at 100 °C in the same equipment without using vacuum pump function. All the cooking experiments were done in duplicate for each operating conditions.

**Brix analysis**

The soluble dry matter of the strawberry jam was measured by Abbe refractometer at room temperature (Cemeroğlu, 2010).

**Color analysis**

The color of the strawberry jam is determined with CIE Yxy, L* a* b* values. Besides, the color intensity (Chroma, C*) and the color tone (Hue, h°) were calculated using equations 1 and 2, respectively (Hunter, 1975; CIE, 1978). Colour measurements were performed with a Minolta Chromameter (Konica Minolta, Osaka, Japan).

Color intensity, Chroma

$$C^* = (a^{*2} + b^{*2})^{1/2} \tag{1}$$

Color tone, hue

$$h^{\circ} = \arctan(b^* + a^*) \tag{2}$$

**pH analysis**

pH values of the strawberry jam was measured by digital pH meter ((inoLab pH/Cond 720, WTW, Germany) at room temperature.

**Titratable acidity**

The strawberry jam was homogenized using a blender. The sample weighed 10 g in 100 ml volumetric flask and was added distilled water. After the sample was filtered by filter paper, 20 ml of filtrate was titrated with 0.1 N NaOH. Fenolftalein was used as indicator (Cemeroğlu, 2010). The titratable acidity which was determined as citric acid % (w/v) was expressed as follows (3):

$$Titratable\ acidity, \% = \frac{V \cdot F \cdot E}{M} \cdot 100 \tag{3}$$

where: $V$ – used 0.1 N NaOH, ml; $F$ – factor of NaOH; $E$ – equivalent acid amount of 1 ml 0.1 N NaOH (citric acid: 0.006404); $M$ – sample amount, ml or g.

**Hydroxymethylfurfural (HMF) content**

Hydroxymethylfurfural (HMF) content of the strawberry jam was determined by HPLC. The strawberry jam (5 g), which was homogenized with blender, was diluted with 50 ml distilled water. The sample was filtered through a blue filter paper and then was injected into the chromatograph. To prepare the calibration curve, HMF standard

was diluted to 10–20–30 mg $L^{-1}$ concentration and peak areas were determined. The amount of HMF in sample was quantitatively determined using the calibration curve. The flow rate was 1 ml $min^{-1}$ and the mobile phase was 80% of distilled water and 20% of methanol. The diode array detector was set at 285 nm and $C_{18}$ (15 cm* 4.6 mm) was used (Vorlová et al., 2006).

**Degree of inversion**

The degree of inversion of the strawberry jam was carried out by volumetric Lane-Eynon method (Cemeroğlu, 2010).

**Sensory evaluation**

Sensory evaluation of the strawberry jam included appearance (remaining in all without disintegration), color, consistency in spoon (fluidity), consistency in mouth (being hard or soft for strawberry grains), taste and overall acceptance. Sensory analysis was performed according to Holtz et al. (1984) and Altuğ & Elmacı (2005) with 10 members. All panelists were non-smokers. The intensity of the properties was determined using a 5–point scale (1 being the lowest and 5 the highest).

**Statistical analysis**

All of the measurements were done in triplicates. Results are shown as mean ± standard deviation.

One-way ANOVA test was conducted to determine the effect of cooking time on the atmospheric pressure cooked and vacuum cooked strawberry jam samples. Also Duncan's multiple range test was evaluated to compare the effect of different processing temperature and times on the quality parameters of the strawberry jam samples. The statistical analyses were performed using SPSS (Statistical Package for the Social Sciences, SPSS Chicago, Illinois, USA) software version 15.0.

## RESULTS AND DISCUSSION

The experiments of the strawberry jam carried out under vacuum at 75 °C for 17.5 and 25 min using the developed vacuum cooking equipment prototype were evaluated. The strawberry jam was also produced at atmospheric pressure for 5, 10, 15 and 20 min in comparison with that samples cooked under vacuum. The physical and chemical properties of the strawberry jam produced at atmospheric pressure and under vacuum are given in Table 1 and ANOVA results are given in Table 2.

One of the most important quality parameters of the strawberry jam is an attractive red colour, in addition to its common sweet–sour strawberry flavour and sufficient jam consistency (Wicklund et al., 2005). The color stability of red fruit is affected by temperature, pH, oxygen and sugar content, presence of ascorbic acid and metal ion and undesirable discoloration could be seen in the product as a result of the degradation of color pigment (Withy et al., 1993). Browning reaction of sugar causes darker red color in jam products and this is usually an undesirable color change (Zor, 2007). Significant difference were observed between the L* values of atmospheric pressure cooked jams ($p < 0.05$) and vacuum cooked jam (Table 1).

**Table 1.** The physical and chemical properties of strawberry jam produced at atmospheric pressure and under vacuum

| | Atmospheric cooking | | | Vacuum cooking | | |
|---|---|---|---|---|---|---|
| Temperature (°C) | 100 | | | 75 | | |
| Pressure (atm) | 1 atm | | | 0.39 atm | | |
| Time (min) | 5 | 10 | 15 | 20 | 17.5 | 25 |
| L* | $28.5^{a} \pm 0.0$ | $28.7^{a} \pm 0.1$ | $28.7^{a} \pm 0.2$ | $28.5^{a} \pm 0.0$ | $28.2^{b} \pm 0.1$ | $28.2^{b} \pm 0.0$ |
| a* | $2.6^{ab} \pm 0.6$ | $3.8^{a} \pm 1.2$ | $3.8^{a} \pm 1.5$ | $3.5^{a} \pm 0.1$ | $1.3^{b} \pm 0.0$ | $1.2^{b} \pm 0.0$ |
| b* | $1.5^{ab} \pm 0.4$ | $1.8^{a} \pm 0.4$ | $1.7^{a} \pm 0.5$ | $1.7^{a} \pm 0.1$ | $0.9^{b} \pm 0.0$ | $0.8^{b} \pm 0.0$ |
| C* | $3.0^{ab} \pm 0.7$ | $4.3^{a} \pm 1.2$ | $4.2^{a} \pm 1.5$ | $3.9^{a} \pm 0.2$ | $1.6^{b} \pm 0.0$ | $1.4^{b} \pm 0.0$ |
| h° | $30.4^{a} \pm 0.3$ | $24.6^{b} \pm 1.5$ | $25.1^{b} \pm 2.5$ | $25.3^{b} \pm 0.2$ | $33.2^{ac} \pm 0.1$ | $36.0^{c} \pm 1.2$ |
| Brix | $49.0^{a} \pm 1.4$ | $51.8^{ab} \pm 2.5$ | $56.5^{bd} \pm 2.1$ | $65.8^{c} \pm 1.1$ | $60.0^{d} \pm 0.0$ | $59.0^{d} \pm 1.4$ |
| pH | $3.7^{a} \pm 0.1$ | $3.7^{a} \pm 0.1$ | $3.7^{a} \pm 0.1$ | $3.6^{a} \pm 0.0$ | $3.8^{a} \pm 0.1$ | $3.7^{a} \pm 0.1$ |
| Titratable acidity (citric acid, %) | $0.5^{a} \pm 0.1$ | $0.6^{a} \pm 0.1$ | $0.6^{a} \pm 0.1$ | $0.6^{a} \pm 0.0$ | $0.5^{a} \pm 0.0$ | $0.6^{a} \pm 0.0$ |
| HMF (mg $kg^{-1}$) | $49.4^{a} \pm 2.1$ | $67.8^{b} \pm 0.8$ | $73.0^{b} \pm 2.0$ | $129.0^{c} \pm 6.4$ | $14.4^{d} \pm 1.5$ | $21.4^{d} \pm 1.8$ |
| Reducing sugar (%) | $13.7^{a} \pm 0.5$ | $17.9^{b} \pm 2.0$ | $21.4^{c} \pm 1.3$ | $27.7^{d} \pm 1.0$ | $9.1^{e} \pm 0.1$ | $11.1^{ae} \pm 0.2$ |
| Sucrose (%) | $30.9^{a} \pm 2.3$ | $28.1^{a} \pm 4.5$ | $33.8^{ab} \pm 0.2$ | $32.8^{ab} \pm 2.1$ | $42.2^{c} \pm 3.1$ | $39.0^{bc} \pm 0.1$ |
| Total sugar (%) | $44.6^{a} \pm 1.8$ | $46.0^{a} \pm 2.5$ | $55.2^{b} \pm 1.0$ | $60.5^{c} \pm 1.0$ | $51.3^{d} \pm 3.2$ | $50.1^{d} \pm 0.2$ |

Results are given as mean ± standard deviation.
The different letter in the same row are significantly different ($p$<0.05).

It was found that a* values of strawberry jam cooked at atmospheric pressure and under vacuum were in good agreement with the literature (Kıvrak, 2010). Also C* values of the vacuum cooked jam were compared with the atmospheric pressure ones, a slight increase was determined due to the high temperature exposure of the product at atmospheric pressure. But increase of pigment concentration does not usually have an effect in C* value (Kırca et al., 2007). Because as commented by Wrolstad et al. (2005), a pink and a dark red color could have similar C* values. However hue values of the atmospheric pressure jam were lower compared to the vacuum cooked ones with regard to the change of the red color to darker red color tone with long cooking period. Although a*, b* and C* values of vacuum and atmospheric pressure cooked jams were significantly different ($p < 0.05$), only 5 min atmospheric pressure cooked jam was not significanly different with vacuum cooked ones (Table 1). Also Hue values of 5 min atmospheric pressure cooked and 17.5 min vacuum cooked jams were not significantly different, however other trials were significantly different ($p < 0.05$). According to ANOVA results, it was observed that cooking time had no significant effect on L*, a*, b* and C* values of the jams cooked at atmospheric pressure and under vacuum. Relying on the color of the strawberry jam, appearance of the jam products cooked at atmospheric pressure and under vacuum for different cooking periods are given in Fig. 2.

Certain darkenings can be clearly seen from Fig. 2. with the increase of cooking time at atmospheric pressure although no significant changes can be observed between vacuum cooked jams.

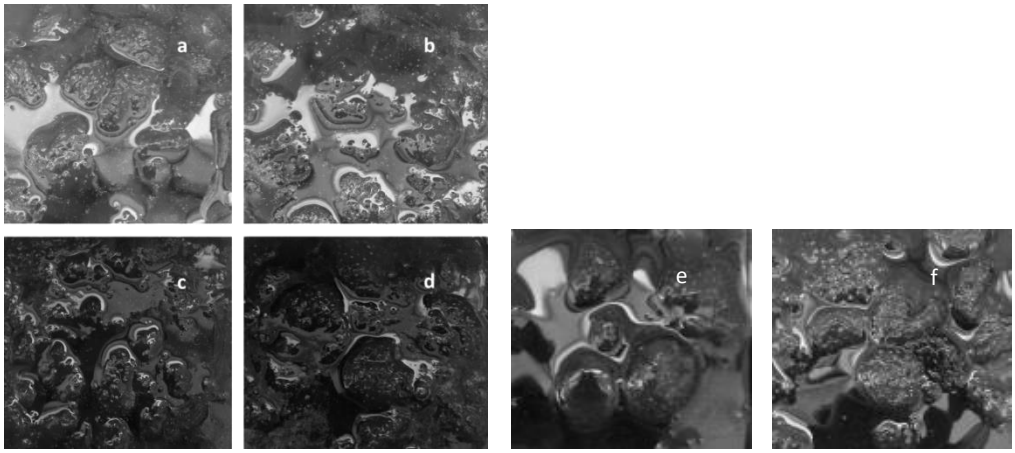


**Figure 2.** Appearance of the strawberry jam products cooked at atmospheric pressure (5,10,15 and 20 min respectively, a, b, c, d) and under vacuum at 75 °C (17.5 and 25 min respectively, e and f).

The Brix values of the strawberry jam products cooked at atmospheric pressure were found to be in the range of 49 and 65.8% and increased with cooking time (Table 1). It was found that cooking time had significant effect on Hue and Brix values of the strawberry jams cooked at atmospheric pressure ($p < 0.05$), however cooking time had no significant effect on Brix values of vacuum cooked ones (Table 2). No significant difference was observed in the jam products cooked under vacuum at different periods. However 20 min processed atmospheric product had the highest Brix value and were more viscous due to the high temperature application. Brix values of the vacuum and atmospheric pressure cooked jams were significantly different ($p < 0.05$) besides the Brix value of 15 min atmospheric pressure cooked jam was not significantly different to vacuum ones (Table 1).

The pH values of atmospheric pressure jam were determined to be in the range of 3.6 to 3.7. A specified pH range for the jams was stated to be 2.8–3.5 in the Turkish Food Codex. Neither the pH values of the atmospheric pressure jam nor the vacuum jam were in these limits due to insufficient citric acid amount to lower the pH of cooking enviroment and provide gelling. It was observed that cooking time had no significant effect on the pH values of both vacuum and atmospheric pressure cooked strawberry jams ($p > 0.05$) (Table 2).

One of the main purpose of the acidity regulator for jam and marmalade products is to increase specific fruit flavor of the product and the other one is to ensure the formation of the desired gelation (Altuğ et al., 2001). Titratable acidity values (as in citric acid) of vacuum cooked samples were ranged between 0.47 and 0.59%, whereas titratable acidity values of atmospheric pressure jams were found to be in the range of 0.48 and 0.62%. Kıvrak (2010) has reported that titratable acidity values of the commercially produced strawberry jam ranged from 0.34 to 0.57%. Also it was determined that they were slightly lower than García-Viguera et al. (1999) found in their research by using different cultivars of strawberry fruit to produce strawberry jam. The titratable acidity values obtained from this study were in good agreement with literature. Only the titratable acidity of the strawberry jam cooked for 20 min at atmospheric

pressure was determined to be slightly higher due to the reactions that began to progress depending on the length of the cooking time. No significant difference ($p > 0.05$) was observed between the pH and the titratable acidity values of vacuum and atmospheric pressure cooked strawberry jam samples (Table 1). However cooking time had significant effect on the titratable acidity values of the vacuum cooked jams ($p < 0.05$) (Table 2).

When the reducing sugar content of the atmospheric pressure cooked strawberry jam was analyzed, it was observed that the amount of reducing sugar ranged between 13.7%. and 27.3%. Also both sucrose % and the reducing sugar % content was increased with the increasing of cooking time. On the other hand, total sugar content of the atmospheric pressure jam products was determined to be in the range of 60.5% and 44.6%.

Also Mohd Naeem et al. (2015) have determined similar total sugar content in strawberry jam products reported in this study. Brix value and amount of reducing sugar and sucrose determine the rate of crystallization. In literature it has been noted that 30–35% of the total sugar should be reducing sugar in the final product to prevent crystallization (Tosun, 1991). Data obtained from the experiments showed that the applied heat treatment was sufficient to provide the inversion in strawberry jam at atmospheric pressure. It was evaluted that cooking time had significant effect on the total sugar content of the jams cooked at atmospheric pressure ($p < 0.05$) (Table 2).

In vacuum cooking reducing sugar content of the jam was increased with the increasing of cooking time. According to ANOVA results, it was determined that cooking time had significant effect on the reducing sugar content of both atmospheric pressure and vacuum cooked jams ($p < 0.05$). When the jam was evaluated in terms of preventing crystallization, inversion was found to be insufficient for jam cooked under vacuum due to the cooking process applied at low temperature and the short-term. Furthermore, vacuum cooked jam products had lower total sugar content % depending on applied lower temperature. It should be also noted that the addition of sugar during jam-making process could cause the remarkable differences (Igual et al., 2013). Reducing sugar content of the atmospheric pressure cooked and vacuum cooked strawberry jams were significantly different ($p < 0.05$) with each other except for the reducing sugar values of the 5 min atmospheric pressure cooked and 17.5 min vacuum cooked jams (Table 1). In addition, total sugar content of the jams cooked under vacuum and atmospheric pressure were significantly different ($p < 0.05$).

Amount of HMF in fruit juice and concentrate products, jam and jelly products, processed sugar-rich products such as molasses and honey, is used as a criteria showing the intensity of heat applied to the product and the suitability of the storage conditions (Telatar, 1985). In 1$^{st}$ class jam products in Turkey, HMF amount is recommended not to exceed 50 mg kg$^{-1}$, while in 2$^{nd}$ class jam products, it is 100 mg kg$^{-1}$ (Gülpek & Başoğlu, 1989; Bilişli, 1998).

**Table 2 .**ANOVA results of the physical and chemical properties of strawberry jam produced at atmospheric pressure and under vacuum

| | Atmospheric cooking | | | | Vacuum cooking | | |
|---|---|---|---|---|---|---|---|
| | Source | df | Sum of Squares | *p*-value | df | Sum of Squares | *p*-value |
| L* | Between Groups | 3 | 0.031 | 0.400 | 1 | 0.001 | 0.817 |
| | Within Groups | 4 | 0.033 | | 2 | 0.018 | |
| | Total | 7 | 0.064 | | 3 | 0.019 | |
| a* | Between Groups | 3 | 2.099 | 0.593 | 1 | 0.017 | 0.069 |
| | Within Groups | 4 | 3.925 | | 2 | 0.003 | |
| | Total | 7 | 6.024 | | 3 | 0.020 | |
| b* | Between Groups | 3 | 0.073 | 0.904 | 1 | 0.000 | 1.000 |
| | Within Groups | 4 | 0.534 | | 2 | 0.001 | |
| | Total | 7 | 0.606 | | 3 | 0.001 | |
| Ch | Between Groups | 3 | 1.995 | 0.647 | 1 | 0.011 | 0.082 |
| | Within Groups | 4 | 4.408 | | 2 | 0.002 | |
| | Total | 7 | 6.402 | | 3 | 0.013 | |
| h° | Between Groups | 3 | 43.61 | 0.048* | 1 | 7.784 | 0.081 |
| | Within Groups | 4 | 8.554 | | 2 | 1.434 | |
| | Total | 7 | 52.17 | | 3 | 9.218 | |
| Brix | Between Groups | 3 | 324.3 | 0.003* | 1 | 1.000 | 0.423 |
| | Within Groups | 4 | 13.75 | | 2 | 2.000 | |
| | Total | 7 | 338.0 | | 3 | 3.000 | |
| pH | Between Groups | 3 | 0.005 | 0.885 | 1 | 0.005 | 0.479 |
| | Within Groups | 4 | 0.029 | | 2 | 0.012 | |
| | Total | 7 | 0.033 | | 3 | 0.017 | |
| Titratable acidity (%) | Between Groups | 3 | 0.019 | 0.464 | 1 | 0.013 | 0.008* |
| | Within Groups | 4 | 0.024 | | 2 | 0.000 | |
| | Total | 7 | 0.043 | | 3 | 0.013 | |
| HMF (mg $kg^{-1}$) | Between Groups | 3 | 7077 | 0.001* | 1 | 48.16 | 0.053 |
| | Within Groups | 4 | 198.3 | | 2 | 5.580 | |
| | Total | 7 | 7275 | | 3 | 53.74 | |
| Reducing sugar (%) | Between Groups | 3 | 212.7 | 0.002* | 1 | 3.725 | 0.004* |
| | Within Groups | 4 | 6.867 | | 2 | 0.032 | |
| | Total | 7 | 219.5 | | 3 | 3.757 | |
| Sucrose (%) | Between Groups | 3 | 35.97 | 0.408 | 1 | 10.36 | 0.286 |
| | Within Groups | 4 | 38.99 | | 2 | 9.936 | |
| | Total | 7 | 74.95 | | 3 | 20.30 | |
| Total sugar (%) | Between Groups | 3 | 345.5 | 0.002* | 1 | 0.053 | 0.858 |
| | Within Groups | 4 | 11.52 | | 2 | 2.591 | |
| | Total | 7 | 357.1 | | 3 | 2.644 | |

* Significant differences in 0.05 level.

HMF content of atmospheric pressure cooked jam products varied from 49.35 mg $kg^{-1}$ to 129.01 mg $kg^{-1}$. It has increased with the cooking time as stated by Eichner (1973) HMF formation in food depended on the reducing sugar and amino acid concentration, pH value of food and process conditions in terms of temperature and time. In our study, it was also observed that cooking time had significant effect on the HMF content of the strawberry jams cooked at atmospheric pressure ($p < 0.05$). Ekşi & Velioğlu (1990) reported that reducing sugar content and HMF content (mg $kg^{-1}$) of jam

products was highly corraleted with each other. HMF content of the vacuum cooked jam products was found to be in the range of 14.41 and 21.35 mg $kg^{-1}$. As stated earlier, HMF content of vacuum cooked strawberry increased slightly with cooking time. Also HMF content of the strawberry jam cooked at atmospheric pressure were excessively high (49.35 to 129.01 mg $kg^{-1}$) than the jam cooked under vacuum. HMF content of the jams cooked under vacuum and atmospheric pressure were found to be significantly different ($p < 0.05$) (Table 1). Vorlová et al. (2006) reported that high temperature appliance has caused Maillard reaction and caramelization depending on the high carbohydrate content and low pH of the product.

Results of the sensory properties of appearance, color, consistency in spoon, consistency in mouth, taste and overall preferences of the strawberry jam produced at atmospheric pressure and under vacuum were shown in Table 3.

**Table 3.** Sensorial evaluation results of the atmospheric pressure and vacuum cooked strawberry jam

| | Atmospheric pressure cooking | | | | Vacuum cooking | |
|---|---|---|---|---|---|---|
| Temperature (°C) | 100 | | | | 75 | |
| Pressure (atm) | 1 atm | | | | 0.39 atm | |
| Time (min) | 5 | 10 | 15 | 20 | 17.5 | 25 |
| Appearance | 3.6 ± 0.6 | 3.8 ± 0.3 | 3.8 ± 0.3 | 3.2 ± 0.1 | 4.2 ± 0.1 | 4.2 ± 0.1 |
| Color | 3.9 ± 0.4 | 4.3 ± 0.0 | 4.0 ± 0.1 | 3.8 ± 0.3 | 4.4 ± 0.2 | 4.4 ± 0.1 |
| Consistency in spoon | 3.6 ± 0.4 | 4.1 ± 0.2 | 3.8 ± 0.3 | 3.8 ± 0.5 | 4.3 ± 0.3 | 4.3 ± 0.1 |
| Consistency in mouth | 3.7 ± 0.3 | 4.1 ± 0.1 | 3.9 ± 0.1 | 3.7 ± 0.5 | 4.2 ± 0.1 | 4.2 ± 0.4 |
| Taste | 3.8 ± 0.4 | 4.0 ± 0.3 | 3.8 ± 0.3 | 3.3 ± 0.9 | 4.3 ± 0.1 | 4.0 ± 0.1 |
| Overall preference | 3.8 ± 0.2 | 4.1 ± 0.1 | 3.8 ± 0.1 | 3.5 ± 0.5 | 4.3 ± 0.3 | 4.2 ± 0.3 |

Whereas consistency in spoon specifies the fluidity of the strawberry jam, the consistency in mouth was associated with being soft or hard of strawberry fruit in jam. While consistency in spoon scores was decreased due to highly viscous structure of jam and consistency in mouth scores was decreased due to being very soft of fruit after 10 min cooking. With the increase in cooking time, color of the jam have darkened, strawberry grains are scattered and had an undesirable appearance. These changes have affected the taste of the jam and has led to low scores given by the panelists. Sensory analysis results showed that the jam cooked for 10 minutes at atmospheric pressure had the highest overall preference score in terms of color, consistency in spoon, consistency in mouth and taste. As seen in Table 2, whereas 17.5 min at 75 °C cooked jam got the highest score in terms of consistency in mouth, taste and overall acceptance, 25 min at 75 °C cooked jam got the highest scores in terms of appearance, color, and consistency in spoon. No significant difference was observed between the sensory properties of vacuum cooked jam except for the taste results. When vacuumed jam products are compared with atmospheric ones, vacuum cooked ones got the highest scores with regard to apperance, color, consistency in spoon, consistency in mouth, taste and overall acceptance.

## CONCLUSION

In this research, a vacuum cooking equipment prototype was designed to work both under vacuum and atmospheric pressure. Brix value indicates the microbiologically resistance and sensorial consistency of the product . Only the Brix of the strawberry jam produced at atmospheric pressure for 20 min was higher (>60) and were more viscous depending on the applied high temperature compared to the other jam products. It was observed that vacuum process was sufficient to acquire the similar Brix value to atmospheric process with a moderate heat treatment. Hue (h°) values of the vacuum cooked jam products were higher compared to atmospheric cooked jams. This result shows that strawberry jam cooked under vacuum protected the color of red fresh strawberry fruit better whereas high temperature applications at atmospheric pressure caused discoloration in the product. Hydroxymethylfurfural (HMF) content of the strawberry jam produced under vacuum was found to be ensured the limit range for 1st class jam products in the literature (<50 mg $kg^{-1}$). Although the HMF content of the atmospheric processed jam for 5 min was under 1st class jam limit (<50 mg $kg^{-1}$), HMF content of the other atmospheric processed jam was found to be exceeding the limit. As it was foreseen at the beginning of the study, vacuum cooking process has been succesfully reduced the HMF content of the strawberry jam due to the low temperature application and oxygen free environment. However it was determined that vacuum cooking application found to be insufficient in providing the inversion compared with atmospheric cooking application. This could lead to crystallisation problems in jam when stored for a long time. Vacuum cooked strawberry jam had the highest overall preference scores by the panelists in terms of attractive fresh strawberry fruit color and less crushed strawberry grains.

In conclusion, this study has shown that appearance and color were better protected, formation of the harmful components such as HMF was decreased and soluble dry matter content was found to be close to the jam produced at traditional ways by vacuum cooking application. Vacuum cooking could be an alternative way to traditional methods at home and this could improve the properties of strawberry jam. This developed prototype could meet the consumers' demand on preparing food with healtier method under their own control at home environment. Furthermore, these findings could also be utilized to improve and develop more household cooking equipment to address the needs of consumers.

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# The temperature changes of barley malt during its disintegration on a two roller mill

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**Abstract.** This article discusses the change of temperature during disintegration on two roller mill. The production of barley and its treatment to malt is first step of beer brewing. The malt is the produced in malt plants. Crushing the malt is realized in only the brewery and is a core activity of brewing technology. This operation is performed both in craft breweries and in industrial brewery. This article therefore is focused on the theory of crashing solid phase with a respect of development of heat. Grinding barley malt is realised using mills of various designs or disintegrators. They are widely used mills with 2, 4, 5, 6 grinding cylinders. These roller mills are used in many other applications, not only in industrial food or drink production. For next treatment solid phase should be broken into smaller pieces (comminuted). The greater the extent of comminution, the large the surface area for impact on next treatment. The amount of mechanical energy converted to heat energy depends on the principle of the process disintegration and other parameters, i.e. distance of grinding gap, capacity, mechanical properties of crashing material etc. For these reasons, it is important to pay attention to the temperature change of barley malt during its disintegration on a mill.

**Key words:** temperature, barley malt, two roller mill, grinding cylinders, disintegration.

## INTRODUCTION

Machines based on the principle of grain grinding by means of rollers are the most frequent grinding machines used in grain mills (it concerns so-called grinding rolling mills) and their derived constructions are used in a number of other food processing industrial sectors (Smejtková & Chládek, 2012). Roller crushers (mills) can be also used in beer production technologies for malt crushing at the very beginning of the beer production. The main working bodies of roller crushers are milling rollers the surface of which is adjusted in various ways. When crushing particles between the rollers, pressure, friction and cutting forces are applied. The grist is usually transported to the grinding space by means of feeding equipment, most frequently equipped with rollers. Grinding roller machines usually consist of a box, feeding equipment, storing equipment (side and central), grinding rollers equipped with roller surface cleaning equipment, gears and motor (Vaculík et al., 2010). Folding equipment enables adjusting of the grinding joint and of the parallel position of rollers (Chládek et al., 2013, Přikryl et al. 2015).

## MATERIAL AND METHODS

The crushing process takes part in the grinding space where the grain is held and processed. Subsequently its fragments are passed through the place where the rollers get the closest together, by the so-called grinding gap. The size of the roller averages affects the grinding space area (Kulp & Ponte, 2000). The crushing intensity can be effectively regulated by changing the gap size. The grinding performance is affected by the length of the rollers and this length must be adjusted so that the rollers are not too long, as in such case they would be subject to deformation (deflexion) (Dendy & Dobraszczyk, 2001; Kalnina et al., 2015). The roller surface finish is grooved, the polished rollers being grooved on special grooving machines. Grinding executed by grain processing with grooved rollers in a mill is a kinetic process during which the grains are held by a low speed (holding roller) and processed by a high roller (crushing roller) with a greater peripheral speed (it concerns a frequent construction of the machine when the grinding rollers in the crusher rotate against each other with differing peripheral speed) (Vaculík et al., 2013). This process is affected by a number of factors ensuing from geometrical and kinematic characteristics, as well as from physical characteristics of the barley malt, which can be defined only with difficulty. The crusher works in a complex way mainly at the level of the grinding gap (Kent & Evers, 1994). The malt grains entering the grinding space are exposed to forces originating on the surface of the rollers and also affecting the other parts of the two-roller mill. The determination of the wattage is based on the precondition of a force $F_B$ passing through centre of gravity, which originates on the surface of the rollers and is applied in the axis passing through the centre of gravity of the rolling space. This axis is parallel with the axis passing through the centres of the rollers. For a horizontal setting of the roller axes the centre of gravity is located in $\delta$ distance. Pressure applied by $F_B$ force on the surface of the rollers creates $F_B$. $\delta$ moment acting against the direction of the roller rotation (Fig. 2) (Maloun, 2001).

To monitor the heat changes in barley malt during crushing using a two-roller mill we selected two-roller mill (crusher) KVM 130/150, thermal recorder IR FlexCam Ti35 and digital thermometer COMMETER D3121.

The basic characteristic of the used barley malt is as follows. It is a product made of barley after four- or five-week maturation in containers. The malt production technology includes at the beginning pre-cleaning of barley followed by barley steeping in special tanks, so-called steep tanks, barley germination and kilning (i.e. drying) of the germinated malt in a kiln. Such germinated but still green malt is first dried by air 60 °C (final water content 3–4%). After the kilning procedure termination dried malt is cleansed from damaged grains, dust and roots and transported to a container in which it must rest before the subsequent processing for a certain period. The basic technical parameters of two-roller mill KVM 130/150 (Fig. 1) are as following: two-roller mill; machine performance (0.044 kg $s^{-1}$); width of roller gap (0.4 mm); measurable specific heat of barley malt ($c_{malt}$ = 1.35 kJ $kg^{-1}$ $K^{-1}$. The specific heat of barley malt $c_{malt}$ was measured in our laboratory calorimeter five times average value was 1.35 kJ $kg^{-1}$ $K^{-1}$. Literature (Manger, H.-J, 1999) specific heat of barley with humidity 0& indicated 1.55 kJ $kg^{-1}$ $K^{-1}$; standard deviation ±2%. The correlations of theoretical and experimental values are very good.

The basic technical parameters of thermal recorder IR FlexCam Ti35 are as follows: high temperature sensitiveness to display even the slightest temperature differences (≤ 0.1ºC); temperature range suitable for a wide choice of industrial applications (-20 °C to +350 °C); flexible lenses movable in the extent of 180º to view pictures in any situation; a large 125 mm contract colour LCD display.

**Figure 1.** Malt mill KVM 130/150 (author's archive).

The basic technical parameters of digital thermometer COMMETER D3121 (with an external probe on the cable) designated for measurement and recording of temperatures are as follows: temperature range (-30 °C up to +105 °C); great thermal sensitivity (0.1 °C) and accuracy (± 0.4 °C).

The torque moment is described as:

$$M_0 = F_B \cdot \delta \tag{1}$$

where: $M_0$ – torque moment (N m); $F_B$ – is the force passing through the centre of gravity (N); $\delta$ – distance of the applied force passing through the centre of gravity $F_B$ from the centre of gravity of the grinding roller axes (m).

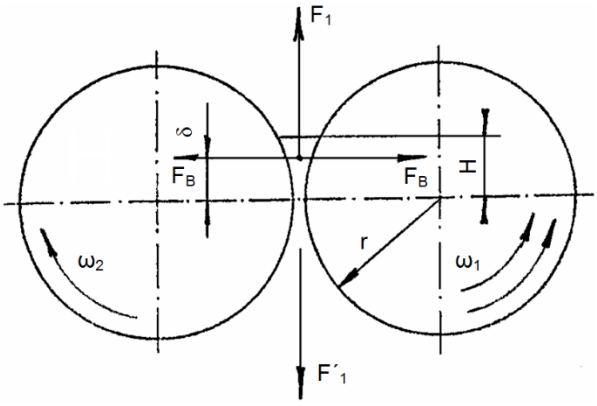


**Figure 2** Grinding rollers and force passing through the centre of gravity (Maloun, 2001): $F_B$ – force passing through the centre of gravity (N); $F_1'$ – resulting tangential force applied on the low-speed (holding) roller (N); $F_1$ – resulting tangential force applied on the high-speed (crushing) roller (N); $r$ – grinding roller radius (m); $H$ – grinding gap height (m); $\delta$ – distance of the applied force passing through the centre of gravity from the centre of gravity of the grinding roller axes (m); $\omega_1$ – angular velocity of the high-speed roller (rad $s^{-1}$), $\omega_2$ – angular velocity of the low-speed roller (rad $s^{-1}$).

Tangential force $F_d$ is described as:

$$F_d = F_B \cdot \frac{\delta}{r} \tag{2}$$

where: $F_d$ – tangential force (N); $F_B$ – force passing through the centre of gravity (N); $\delta$ – distance of the applied force passing through the centre of gravity from the centre of gravity of the grinding roller axes (m); $r$ – roller radius (m).

The high speed roller rotates in opposite direction as low speed one with differing peripheral speeds. When the malt grist passes through the grinding space, another tangential force $F_t$ is originating due to the friction of the grist fragments on the roller cutting edges. In compliance with the force definition it can be expressed as follows:

$$F_t = F_B \cdot tg\ \varphi \tag{3}$$

where: $F_t$ – tangential (friction) force (N); $F_B$ – force passing through the centre of gravity (N); $\varphi$ – friction angle (°).

Forces $F_d$ and $F_t$ are summed up algebraically. It ensues from the theoretic analysis of the originating mechanical tensions that the pressure of force $F_B$ originating on the entire surface of the ground material (along the length of roller $L$) affects the diameter of the rollers with radius $r$ by means of tangential force $F_d$ applied against their rotation. The moving of the grains in the high speed roller is slowed down by means of the low speed roller (with slower rotation) on the entire surface of the ground material by means of tangential (friction) force $F_t$. The resulting tangential force applied on the grinding high speed roller can be expressed as:

$$F_1 = F_B \cdot tg\ \varphi + F_B \cdot \frac{\delta}{r} = F_B \cdot \left( tg\ \varphi + \frac{\delta}{r} \right) \tag{4}$$

where: $F_1$ – resulting tangential force applied on the high-speed (crushing) roller (N); $F_B$ – force passing through the centre of gravity (N); $\varphi$ – friction angle (°); $\delta$ – distance of the applied force passing through the centre of gravity from the centre of gravity of the grinding roller axes (m); $r$ – roller radius (m).

The resulting force ion low speed roller is described as:

$$F_1' = F_B \cdot tg\ \varphi - F_B \cdot \frac{\delta}{r} = F_B \cdot \left( tg\ \varphi - \frac{\delta}{r} \right) \tag{5}$$

where: $F_1'$ – resulting tangential force applied on the low speed (holding) roller (N); $F_B$ – force passing through the centre of gravity (N); $\varphi$ – friction angle (°); $\delta$ – distance of the applied force passing through the centre of gravity from the centre of gravity of the high speed roller axes (m); $r$ – grinding roller radius (m) (Maloun, 2001; Feynman et al., 2011).

The torque moment ensuing from the compound forces applied on the high speed roller:

$$M_1 = F_B \cdot r \cdot \left( tg\ \varphi + \frac{\delta}{r} \right) \tag{6}$$

where: $M_1$ – torque moment ensuing from the compound forces applied on the high speed roller (N m); $F_B$ – is the force passing through the centre of gravity (N); $\varphi$ – friction angle (°); $\delta$ – distance of the applied force passing through the centre of gravity $F_B$ from the centre of gravity of the high speed roller axes (m); $r$ – high speed roller radius (m).

The torque moment $M_2$ passes from the low speed roller to the high speed roller describes as:

$$M_2 = \frac{F_B \cdot r \cdot \left( tg\ \varphi - \frac{\delta}{r} \right)}{K} \tag{7}$$

$$K = \frac{v_{hs}}{v_{ls}} \tag{8}$$

where: $M_2$ – torque ensuing from the compound forces applied on the crushing roller (N.m); $F_B$ – is the force passing through the centre of gravity (N); $r$ – grinding roller radius (m); $\varphi$ – friction angle (°); $K$ – ratio $v_{hs}/v_{ls}$ ( – ); $\delta$ – distance of the applied force passing through the centre of gravity $F_B$ from the centre of gravity of the grinding roller axes (m); $v_{hs}$ – peripheral velocity of the high speed roller (m.s$^{-1}$); $v_{ls}$ – peripheral velocity of the low speed roller (m.s$^{-1}$).

When processing the malt grains the high speed roller requires that the torque moment of the mill has the following value:

$$M_3 = M_1 - M_2 \tag{9}$$

where: $M_3$ – torque moment of the mill (N m); $M_1$ – torque moment ensuing from the compound forces applied on the crushing roller (N m); $M_2$ – torque moment passes from the low speed roller to the crushing roller (N m).

At the angular speed of the high speed roller of $\omega_1$ the performance of the electromotor described as:

$$P_w = M_3 \cdot \omega_1 \tag{10}$$

where: $P_w$ – power of electomotor (W); $\omega_1$ – angular velocity of the high speed roller (rad s$^{-1}$); $M_3$ – torque moment of the mill (N m).

The performance needed to drive the rollers can be determined based on the following relation:

$$P_w = \frac{\pi}{30} \cdot \left[ T \cdot r \cdot \left( n_1 - n_2 \right) + N \cdot \delta \cdot \left( n_1 + n_2 \right) \right] \tag{11}$$

$$n_1 = \frac{30 \cdot \omega_1}{\pi} \tag{12}$$

$$n_2 = \frac{30 \cdot \omega_2}{\pi \cdot K} \tag{13}$$

where: $T$ is $T = F_B . tg\ \varphi$ (N) and $N = F_B$ (N); $n_1$ – number of rotations of the high speed roller (min$^{-1}$); $n_2$ – number of rotations of the low speed roller (min$^{-1}$); $\varphi$ – friction angle (°); $K$ – ratio $v_{hs}/v_{ls}$ ( – ); $\delta$ – distance of the applied force passing through the centre of

gravity $F_B$ from the centre of gravity of the grinding roller axes (m); $\omega_1$ – angular velocity of the high speed roller (rad $s^{-1}$); $\omega_2$ – angular velocity of the low speed roller (rad $s^{-1}$).

The performance of the roller mills can be determined according to the following relations, while the theoretic mass flow of the grist through the roller mill can be determined as:

$$R = s \cdot l \cdot v_{str} \cdot \rho \cdot \psi \tag{14}$$

$$v_{str} = \frac{v_r + v_p}{2} \tag{15}$$

where: $s$ – grinding gap (m); $l$ – length of rollers (m); $v_{str}$ – medium speed of the grist in the grinding gap (m $s^{-1}$); $\rho$ – volume weight of the grist (kg $m^{-3}$); $\psi$ – coefficient of the grinding space filling ( – ); $v_r$ – peripheral velocity of the high speed roller (rad $s^{-1}$); $v_p$ – peripheral velocity of the low speed roller (rad $s^{-1}$).

According natural laws two bodies not chemically affecting each other balance their temperatures upon mutual contact. This phenomenon can be explained in the way that a certain value of heat passes from the substance with a higher temperature to the substance with a lower temperature and both temperatures equilibration. When the temperature of two interfering bodies levels off, the resulting temperature does not represent an average of both temperatures; not even in the case when we take into account the weight of such bodies. It has occurred that different times are needed when using the same heater to heat the same amount of different substances to the same temperature. Therefore the term thermal amount $Q$ has been established, which is defined as the product of weight $m$, constant $c$ and temperature $T$ (while according to the law of energy conservation at the resulting temperature $t$ the heat accepted by the cooler body must equal the heat surrendered by the warmer body).

The heat transfer from bodies with higher temperature to the surrounding with lower temperature occurs in three ways: by conduction, convection and radiation. Conduction spreads heat in a substance which can have a solid, liquid or gaseous form. During this heat transfer molecules in places with higher temperature have a greater kinetic energy which is partially surrendered to the neighbouring molecules without any moves in the surrounding (free electrons contribute to this transfer in metals).

The amount of heat $Q$ passing through area $S$ for time $\tau$ in a substance with thickness $d$, provided that a fixed temperature differential is maintained on both sides of the layer ($t - t_0$), is established by the following relation:

$$Q = \lambda \cdot S \cdot \tau \cdot \frac{t - t_0}{d} \tag{16}$$

where: $Q$ – the heat needed for heating (J); $S$ – area ($m^2$); $\tau$ – time (s); $\boldsymbol{\lambda}$ – thermal conductivity of the given substance (J $m^{-1}.s^{-1}$) ; $t$ – final temperature ( °C); $t_0$ – initial temperature ( °C); $d$ – thickness (m).

Thermal conductivity represents the amount of heat which passes through the area of 1 $m^2$ per one second at the temperature differential of 1 °C per 1 m.

$$Q = m \cdot c \cdot \Delta T \tag{17}$$

$$\Delta T = T_2 - T_1 \tag{18}$$

where: $Q$ – the heat needed for heating (J); $m$ – weight of the malt sample (kg); $c$ – specific heat (J $kg^{-1}$ $K^{-1}$); $\Delta T$ – temperature difference (K); $T_2$ – final temperature of the malt sample (K); $T_1$ – initial temperature of the malt sample (K).

The arithmetic average was used for evaluation of the measurement. The arithmetic average is defined as being equal to the sum of the numerical values of each and every observation divided by the total number of observations. Symbolically, if we have a data set containing the values $a_1$... $a_n$. The arithmetic average is defined as:

$$\phi = \frac{1}{n}\sum_{i=1}^{n} a_i \tag{19}$$

where: $\emptyset$ – arithmetic average ( – ); $a_1$,..., $a_n$ – the values of data set (Maloun, 2001; Feynman et al., 2011).

## RESULTS AND DISCUSSION

The Figs 3 and 4 provide pictures performed by the thermal recorder during barley malt grinding at two-roller mill KVM 130/150.

The following table (Table 1) shows the measured temperature values during barley malt grinding on two-roller mill KVM 130/150 in the course of five measurements. The provided values of the particular temperatures were measured before the grinding initiation (i.e. time 0 min.) during grinding of 13.3 kg of grist (i.e. 5 minutes after the grinding initiation) and at fixed temperatures, which corresponds to grinding of 160 kg of grist (i.e. in 60 minutes).

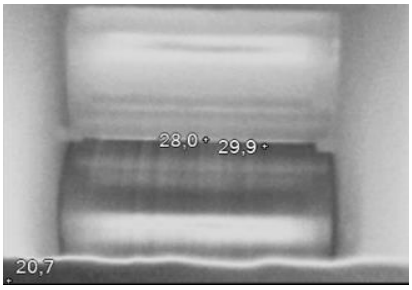


**Figure 3.** Temperatures of grinding rollers (60 min of grinding) (author's archive).

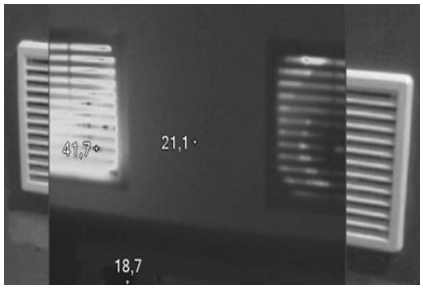


**Figure 4.** Temperatures of the mill outer surface (60 min of grinding) (author's archive).

**Table 1.** Measured temperature values during barley malt grinding on two-roller mill KVM 130/150

| Measurement number | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Temp. (°C) | T* | TR* | **Ø1*** | T* | TR* | **Ø2*** | T* | TR* | **Ø3*** | T* | TR* | **Ø4*** | T* | TR* | **Ø5*** |
| Air in the room | 18.0 | 18.0 | **18.0** | 19.0 | 19.0 | **19.0** | 18.0 | 18.0 | **18.0** | 19.5 | 19.5 | **19.5** | 18.5 | 18.5 | **18.5** |
| Time 0 minutes (i.e. before grinding initiation) | | | | | | | | | | | | | | | |
| Machine external | 18.0 | 18.0 | **18.0** | 19.0 | 19.0 | **19.0** | 18.0 | 18.0 | **18.0** | 19.5 | 19.5 | **19.5** | 18.5 | 18.5 | **18.5** |
| Machine rollers | 18.3 | 18.9 | **18.6** | 19.4 | 19.8 | **19.6** | 18.6 | 19.0 | **18.8** | 20.0 | 20.2 | **20.1** | 18.7 | 18.3 | **18.5** |
| Malt before crushing | 19.4 | 18.8 | **19.1** | 18.8 | 19.3 | **19.1** | 17.7 | 17.8 | **17.8** | 19.4 | 19.2 | **19.3** | 19.4 | 19.6 | **19.5** |
| Time +5 minutes (i.e. 5 minutes of crushing = 13.3 kg grist) | | | | | | | | | | | | | | | |
| Malt after crushing | 20.9 | 20.2 | **20.6** | 21.1 | 20.9 | **21.0** | 20.8 | 21.0 | **20.9** | 21.9 | 21.7 | **21.8** | 20.6 | 20.4 | **20.5** |
| Machine rollers | 20.6 | 20.6 | **20.6** | 21.5 | 21.3 | **21.4** | 20.8 | 20.4 | **20.6** | 22.2 | 22.4 | **22.3** | 21.0 | 20.9 | **21.0** |
| Barley malt in hopper | 18.8 | 18.4 | **18.6** | 18.8 | 19.0 | **18.9** | 19.0 | 18.6 | **18.8** | 20.0 | 20.0 | **20.0** | 18.8 | 18.4 | **18.6** |
| Temperature differential of malt and grist after 5 minutes of grinding | | | | | | | | | | | | | | | |
| | - | - | **2.0** | - | - | **2.1** | - | - | **2.1** | - | - | **1.8** | - | - | **1.9** |
| **Arithmetic average of temperature different** | | | | | | | | | | | | | | | **1.98** |
| Time +60 min (i.e. 60 min of crushing = 160 kg grist) (settled temperature) | | | | | | | | | | | | | | | |
| Malt after crushing | 21.4 | 21.2 | **21.3** | 21.5 | 21.3 | **21.4** | 21.3 | 21.6 | **21.5** | 22.6 | 22.4 | **22.5** | 21.2 | 21.6 | **21.4** |
| Machine rollers | 28.1 | 27.9 | **28.0** | 27.8 | 28.0 | **28.0** | 28.4 | 28.0 | **28.2** | 29.5 | 30.1 | **29.8** | 27.3 | 27.5 | **27.4** |
| Barley malt in hopper | 17.8 | 18.2 | **18.0** | 17.6 | 18.0 | **17.8** | 17.8 | 18.2 | **18.0** | 19.7 | 19.3 | **19.5** | 18.0 | 17.8 | **17.9** |
| Temperature differential of malt and grist after 60 minutes of grinding at fixed temperature | | | | | | | | | | | | | | | |
| | - | - | **3.3** | - | - | **3.6** | - | - | **3.5** | - | - | **3.0** | - | - | **3.5** |
| **Total arithmetic average of temperature different** | | | | | | | | | | | | | | | **3.38** |

*Explanatory notes:
T – thermometer; TR – thermal recorder; Ø – arithmetic average of measurement values.

Experimental measured values have been evaluated statistically using program in computer.

Theoretical calculation of the theoretical heat needed for heating and the measured heat needed for heating as follows from the equation 16:

$$Q_1 = m \cdot c_{malt1} \cdot \Delta T = 160 \cdot 1{,}55 \cdot 3{,}38 = 838{,}24\,K \qquad (20)$$

$$Q_2 = m \cdot c_{malt2} \cdot \Delta T = 160 \cdot 1{,}35 \cdot 3{,}38 = 730{,}08\,K \qquad (21)$$

where: $Q_1$ – the theoretical heat needed for heating (J); $Q_2$ – the measured heat needed for heating (J); $m$ – weight of the barley malt sample (kg); $c_{malt1}$ – theoretical specific heat of barley malt (J kg$^{-1}$ K$^{-1}$); $c_{malt2}$ – measured specific heat of barley malt (J kg$^{-1}$ K$^{-1}$); $\Delta T$ – temperature difference (K) (Manger, H.-J, 1999; Feynman et al., 2011).

## CONCLUSION

When grinding using blows (i.e. when grinding by means of hammer crushers that are also used to process barley malt for the beer production) less than 5 percents of wattage are used for the disintegration itself, while 95% are not related with disintegration at all; it is wasted energy dissipating into heat and causing heating of the ground material and/or the machine body. When using the same material to be ground – barley malt and with the same parameters of the input product the grist after grinding on the hammer crusher was heated up by 8.5 K to 15 K (according to the type of crusher used). By measuring the barley malt grinding on two-roller mill KVM 130/150 we found out that the average heating of the resulting grist (at fixed temperature values) reaches 3.38 K.

The obtained experimental results are in good correlation with theoretical assumption, as shown in table 1.

This value (with the measurable energy consumption of 4.46 kJ $kg^{-1}$) is not significant, as regards influencing of the grinding process, the technical parameters of the mill and the resulting characteristics of the grist, and it confirms that using of roller mills for the malt crushing while maintaining the required characteristics of the resulting grist (the grist structure) is beneficial as concerns both the consumed energy and the amount of dissipated heat. The performed measurements of the barley malt temperature changes during crushing on two-roller mill were carried out as a part of the overall assessment of the malt grinding problematic.

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# Modeling of impact parameters for nondestructive evaluation of firmness of greenhouse tomatoes

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**Abstract.** In this research, the potential of a nondestructive method for predicting firmness using impact parameters taken by a low-mass lateral impact device was explored. The tests were carried out on *Bandita F1* greenhouse tomato variety at different maturity stages. In the nondestructive impact measurements, impact acceleration and contact time were sensed by an accelerometer attached on impact head, and main impact parameters such as maximum impact acceleration (A), time required to reach maximum acceleration (t) and contact time ($t_c$) were extracted from the impact acceleration-contact time curves. Other impact parameters were derived through the theory of elasticity. These nondestructive impact parameters were compared with destructive reference parameters for predicting firmness of tomatoes. Force-deformation ratio at rupture point was used in the measurements of destructive reference parameter and this was expressed to be tomato firmness. A total of 10 ($A, t, t_c$, $A/t$, $A/t_c$, $A/t^2$, $A/t_c^2$, $(1/t)^{2.5}$, $(A/t)^{1.25}$, $A^{2.5}$) measured and derived impact parameters were analyzed with the destructive reference test. A correlation matrix, stepwise regression and multiple linear regression were used for statistically evaluation. The effect of maturity stages on firmness and impact parameters was investigated by ANOVA test. Statistical analysis showed that the correlations between destructive reference and nondestructive impact parameter test results were significant at 1% level except $t$ and $(1/t)^{2.5}$. The number of parameters being processed was reduced with stepwise regression analysis. The best model using MLR on variables $/t, A/t_c^2$, and $A^{2.5}$ was selected for predicting tomato firmness. As a result, low-mass impact device tested in the laboratory conditions gave high prediction of firmness for greenhouse tomato.

**Key words:** greenhouse tomato, nondestructive low-mass impact device, tomato firmness, impact parameters, multiple linear regression.

## INTRODUCTION

For fresh tomatoes, the two quality attributes that are most important to buyers and consumers are texture and skin color (Batu, 2004). Texture is influenced by flesh firmness and skin strength. Softening during storage, distribution and ripening of tomatoes can be a major problem because of the susceptibility to bruise damage. The loss of fruit firmness is a physiological process that occurs during fruit maturation/ripening on the tree, during cold storage and retail handling (Valero et al., 2007). The firmness of a fruit is an index of the mechanical, chemical and rheological

properties of the fruit. It is negatively proportional to the maturity of the fruit, and can therefore be used as an alternative indicator to maturity in fruit grading and sorting (Lien et al., 2009).

Magness-Taylor test, which is called as destructive measurement is a classical method and commonly used for measuring the fruit flesh firmness. This test is conducted by handheld penetrometer or a PC controlled material test device that records the force required to puncture the flesh with a cylindrical probe of fixed diameter and tip geometry. Destructive reference test measures the mechanical attitudes of fruits under the static loading.

At present, some nondestructive techniques such as acoustic, ultrasonic, vibration, micro-deformation, impact and near infrared (NIR) were applied to many fruits and vegetables to evaluate the texture quality (Sirisomboon, 2012). According to these nondestructive detection methods, some commercial firmness sorting device (bench top) or systems (in-line) have been using in practice (Garcia-Ramos et al., 2003).

The firmness of fruits and vegetables is usually managed by the workers in the field through a destructive sampling on several lots: during the conferring of goods, the pre-stocking, the post-stocking, the packaging and before delivery. All these stages need a rather long time and a large waste of fruits; moreover they are not always homogenous. Instead of destructive firmness measurement, nondestructive techniques can satisfy easy and quick use of the system, customers can test bigger sampling within the same lot. This system also avoids the variability that can be caused by manual labor of workers. The nondestructive systems make use of the sensor technology and they can be assembled on existing packing lines. Plocharski & Konopacka (2003) was developed a method based on the measurement of the plums, using a cylindrical probe and a force of 1 N. This method was extremely sensitive and was fully non-destructive. Although this method was non-destructive it was not adopted to a firmness sorting systems because of using the Universal Testing Machine. Previous studies carried out by different researchers show that the nondestructive impact techniques can be used to evaluate firmness of fruits and vegetables successfully (Nahir et al., 1986; Delwiche et al., 1987; Garcia-Ramos et al., 1988; Chen & Ruiz-Altisent, 1993; Diezma-Iglesias et al., 2006; Lien et al., 2009; Ragni et al., 2010).

Two different methods based on theory of elasticity have been used to measure fruit firmness using the nondestructive impact technique. The first one is the force response of an elastic sphere impacting on a rigid surface. A problem inherent to the technique of dropping the fruit on a force sensor is that the impact force is also a function of the mass and radius of curvature of the fruit. Therefore, a large variation in these two parameters will affect the accuracy in firmness measurement (Chen et al., 1996). The second one is to impact the fruit with a small spherical impactor of known mass and radius of curvature and measure the acceleration of the impactor. The advantage of this method is that the measured impact acceleration response is independent of the fruit mass and is less sensitive to the variation of the radius of curvature of the fruit. Many researchers have studied on the impact of fruit nondestructively on a force sensor. Nahir et al. (1986) reported that impact force magnitude substantially is related with fruit mass and fruit firmness in the case of dropping tomato from a 70 mm height on a rigid surface. Delwiche et al. (1987) analyzed impact force response of peach samples striking a rigid surface and found that impact force parameters were closely related with the fruit's modulus of elasticity and fruit flesh firmness. Lien et al. (2009) used nondestructive

impact technique to determine tomato ripeness. They reported that maximum impact force, impact time and fruit mass was related highly with Magness-Taylor force of tomato dropped on a force sensor with a classification precision of 82.30%. Ragni et al. (2010) for kiwi and Gutierrez et al. (2007) for peach also reported successfully nondestructive firmness sorting based on the analysis of fruit impact on a load cell in pre-commercial sorting line.

Chen et al. (1985) first described the impact technique with a small spherical impactor to the fruit, and this technique was used by researchers in Spain for sensing fruit firmness. Further versions have been developed at the Physical Properties Laboratory (LPF) to obtain systems with better data resolution, signal-noise ratio and precision (Diezma et al., 2000). Thereafter, Garcia-Ramos et al. (2003) modified and adapted this lateral impact sensor to a prototype impact system for evaluating on-line firmness sorting of fruits. Dieazma-Iglesias et al. (2006) estimated peach firmness by using nondestructive impact and acoustic tests. For impact and acoustic tests, low-mass lateral impactor developed by researchers was used in their laboratory. As well as using these techniques for determining fruit firmness, some researchers used this technique as a reference test for monitoring fruit ripeness with different methods. For instance, Ruiz-Altisent et al. (2006) studied the feasibility of using nondestructive information such as optical reflectance combined with contact firmness to estimate ripeness and consumers acceptability of peaches at harvest site. Also, Herrero-Langreo et al. (2011) studied spectral machine vision for peach ripeness assessment at harvest and post-harvest, and used Magness-Taylor penetrometry firmness and low-mass impact firmness as a reference measurement.

The objectives of the present research were to determine the relationship between tomato firmness and nondestructive impact parameters, to develop the calibration equation with multiple linear regression using impact parameters and, to explore the potential of prediction of the tomato firmness nondestructively using low-mass impact device.

## MATERIALS AND METHODS

Fresh greenhouse tomatoes (*Bandita F1*) that were sorted by color and size, free from disease and injury, and uniform in shape were harvested by hand from a commercial greenhouse in 2014 season.

Color measurements were performed using Minolta CR-400 colorimeter; four replicates in the equatorial region were taken on each intact tomato. The L*, a* and b* values were obtained directly, and were used to calculate the a*/b* ratio. Average readings at four pre-determined points on the circumference of the fruits were recorded. The instrument was calibrated against a standard white color Plate (Y = 93.5, x = 0.3114, y = 0.3190). In a Minolta chromometer, the a* value corresponds to the degree of redness whereas the b* value represents yellowness. In this research, redness values of tomatoes were recorded as a*/b* values due to the convention of recording tomatoes redness values as a*/b* in the Hunter system for many years (Batu, 2004). Table 1 shows the relationship between the a*/b* ratio and maturity stages of tomatoes.

**Table 1.** a*/b* values used for maturity classification of tomatoes (Batu, 2004)

| Maturity stage | a*/b* |
|---|---|
| Mature green | -0.59<a*/b*≤-0.47 |
| Breaking | -0.47< a*/b*≤-0.27 |
| Turning | -0.27< a*/b*≤0.08 |
| Pink | 0.08< a*/b*≤0.60 |
| Light red | 0.60< a*/b*≤0.95 |
| Red | a*/b*>0.95 |

Tomatoes were classified at six different maturity stages (mature green, breaking, turning, pink, light red and red) according to the a*/b* ratio recommended by Batu (2004).Tomatoes at six maturity stages were used for getting a wide range of firmness stage depending on the maturity properties for destructive and nondestructive measurements. Three major dimensions of tomato fruits were illustrated in Fig. 1.

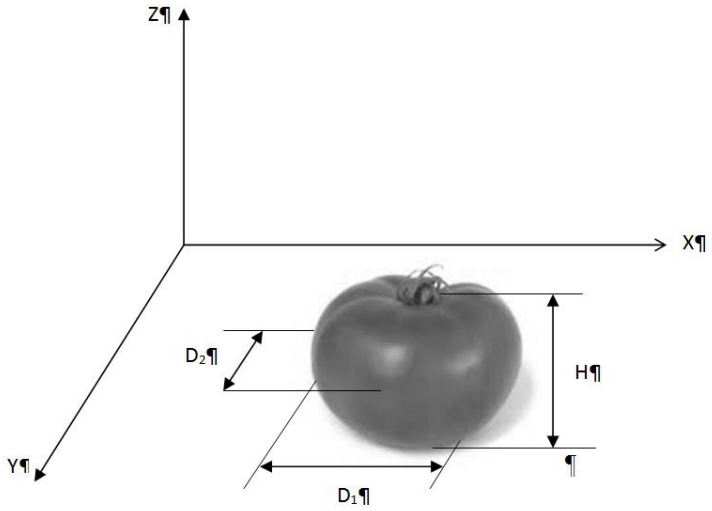


**Figure 1.** Three major dimensions of tomato: D1, Equatorial diameter; D2, Thickness; H, Height.

A nondestructive low-mass lateral impact device, which is similar to test device developed by Chen & Ruiz-Altisent (1996) have been manufactured and used in the experiment. the nondestructive lateral low-mass impact device showing all the main components was given in Fig. 2. It consists of a spherical low-mass of 26 g, which impacts the sample, with a piezoelectric accelerometer of a mass of 1.5g, sensitivity of 1.063 mV $m^{-1}$ $s^2$ and a range of ± 4,900 m $s^{-2}$ (DeltaTron® Accelerometer Type 4516 manufactured by Bruel&Kjaer), which impacts the fruits to sense its firmness; a spring to release the impacting mass; and an electromagnet to hold the impacting mass. Radius of curvature of the semi-spherical impacting mass was designed to be 25 mm as suggested by Van Linden et al. (2006).

At the impactor was held by an electromagnet, it saved potential energy and after releasing, its saved potential energy modified to kinetic energy during the releasing and impacted to tomato samples with a velocity of about 0.28 m $s^{-1}$. Furthermore, impact energy of the lateral impactor during the impact process was calculated to be 1.02 mJ. Due to impactor was designed also considering fruit elasticity threshold, low impact forces were composed (about 2–4 N) during the impact on fruit surface and thus mechanical damage did not occur on the tomato surface. For this reason, measurements by means of impactor were named as 'nondestructive measurement'. The distance between lateral impactor and peach was fixed at 2 cm as suggested by Vursavus et al. (2015). A conditioning circuit (Model 4102C, DYTRAN) supplies power to the accelerometer and also amplifies the acceleration signal. Response of the accelerometer was sampled at 100 kHz sampling rate with 16 bit precision NI 6221PCI DAQ card. A MATLAB based software was designed to control all the process which stores data and provides the users with an interface to manage the data and control the measurement process. Impact acceleration, impact velocity and deformation–contact time curves could be monitored graphically in MATLAB software interface. By means of an accelerometer mounted on impact head, main impact parameters such as maximum acceleration ($A$), measured in m $s^{-2}$, impact duration until maximum acceleration ($t$) in ms and impact duration ($t_c$) in ms were extracted from the deceleration data registered by the accelerometer. These parameters are commonly used as fruit firmness index (Chen & Tjan, 1998). Totally, ten nondestructive impact parameters ($A, t, t_c$, $A/t$, $A/t_c$, $A/t^2$, $A/t_c^2$, $(1/t)^{2.5}$, $(A/t)^{1.25}$, $A^{2.5}$) were used and, $A^{2.5}$, $(A/t)^{1.25}$ and $(1/t)^{2.5}$ were derived by using the theory of elasticity as suggested by Slaughter et al. (2009) and Vursavus et al. (2015) from the main impact parameters such as $A$, $t$ and $t_c$ for modeling of these impact parameters for nondestructive evaluation of firmness of tomatoes. In this study, four replicates in the equatorial region were taken on each intact tomato for nondestructive impact measurements at the same points of the color measurements.

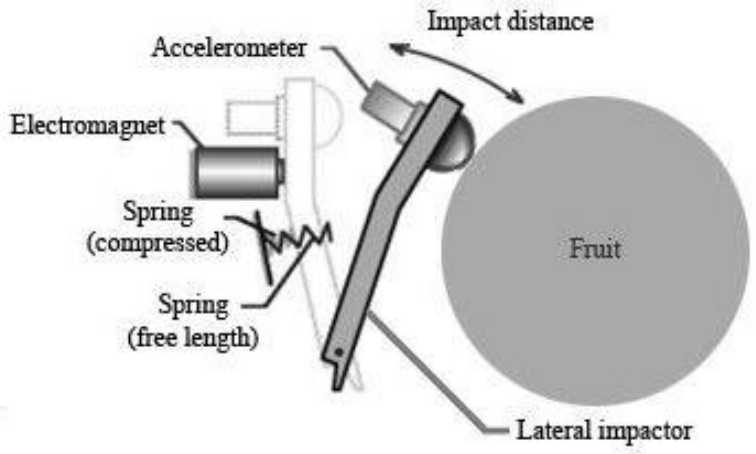


**Figure 2.** Schematic drawing of the low-mass lateral impact device showing all the main components.

The reference destructive tests were conducted to define the firmness stage of tomato samples. Lloyd Testing Machine (Model LRX Plus Series) was used for the mechanical test to determine the firmness group of the test samples and, to compare with the nondestructive impact parameters. Puncture test was performed by using a flat ended probe with 4 mm diameter, at a deformation rate of 10 mm $min^{-1}$ at four equatorial region of each tomato fruit. The load-cell admits a maximum force of 5,000 N (resolution 0.005 N) and an error range of 0.03%. Destructive firmness measurements were taken after nondestructive measurements on exactly the same points as the other measurements. For destructive measurements, on each labeled place, puncture probe penetrated at least 11 mm into the flesh. Force-deformation ratio at maximum point was selected from the force-deformation curve and expressed to be tomato firmness in N $mm^{-1}$ ($F_T$).

A correlation matrix, which gives the correlations between pairs of these ten variables was obtained. Stepwise regression analysis was made on all the impact parameters in order to identity those variables (independent variables) which could be used to predict tomato firmness (dependent variable). After the independent variables were selected, multiple linear regression (MLR) was used to determine the linear relationship of selected parameters to tomato firmness. The average value of each tomato was determined, and the fruit were randomly segregated into calibration and validation sets, with 52 tomatoes in the calibration set and 36 tomatoes in the validation set. The calibration set was used for model development, and the fruit from the validation set were reserved for model testing. MLR analyses were conducted using SPSS Statistics 20 in order to evaluate tomato firmness models. The data recorded in the test conditions were statistically analysed using one way ANOVA to study the effect of tomato maturity stages on tomato firmness and main impact parameters. DUNCAN's multiple range test was used to compare the means.

A total of 88 whole tomato samples were used in the experiments. Four replicates in the equatorial region were taken on each intact tomato. These four data were then averaged for color, nondestructive and destructive tests. Totally, 352 (88 x 4) measurements were recorded in order to use in the statistical analysis.

## RESULTS AND DISCUSSION

The effect of tomato maturity stages on tomato firmness and main impact parameters was determined using one way ANOVA test. As seen in Table 2, tomato firmness decreased from 3.58–1.23 N $mm^{-1}$ significantly during tomato ripening ($P < 0.01$). This implies the softening of the tomato fruit. According to DUNCAN's multiple range test results, tomato firmness in the light red and red stages was not significantly different ($\alpha = 0.05$). Same trend was also observed in the study conducted by Sirisomboon et al. (2012). The main impact parameters $A$, $t$ and $t_c$ give direct information about the firmness of tomato. The effect of maturity stages on $A$ and $t_c$ was found to be statistically significant ($P < 0.01$). This effect was not significant for $t$ parameter. These results showed that $A$ and $t_c$ impact parameters were sensitive parameters related to maturity stages. As seen in Table 2, there are clear differences among impact parameters $A$ and $t_c$ according to maturity stages or fruit softening. Hard mature tomato has a maximum acceleration and minimum impact duration, whereas a

soft red tomato shows opposite results. Furthermore, the $t_c$ can also be used as an effective indicator of firmness. This occurrence comes from that the soft tomatoes (light red and red stages) has a much less firmness than the intermediate (turning or pink stages) and the hard tomatoes (mature green and breaking stages). Hence, this leads to a prolonged total contact time in the soft peaches. As a sample, impact acceleration-contact time curve for a low-mass impact of a rigid sphere on mature green, pink and red tomato samples is shown in Fig. 3.

**Table 2.** Measurements of firmness and main impact parameters to six maturity stages for Bandita F1 tomato varieties

| Para-meters | Maturity stages | | | | | |
|---|---|---|---|---|---|---|
| | Mature green | Breaking | Turning | Pink | Light red | Red |
| $F_T$ | $3.58 \pm 0.10^{a}$ | $2.91 \pm 0.15^{b}$ | $2.21 \pm 0.13^{c}$ | $1.72 \pm 0.08^{d}$ | $1.36 \pm 0.11^{e}$ | $1.23 \pm 0.08^{e}$ |
| $A$ | $377.27 \pm 27.93^{a}$ | $355.35 \pm 17.46^{a}$ | $314.84 \pm 22.55^{b}$ | $303.97 \pm 25.84^{c}$ | $290.15 \pm 29.58^{d}$ | $270.29 \pm 38.47^{d}$ |
| $t$ | $2.06 \pm 0.19^{a}$ | $2.09 \pm 0.17^{a}$ | $2.17 \pm 0.26^{a}$ | $2.09 \pm 0.13^{a}$ | $2.09 \pm 0.13^{a}$ | $2.20 \pm 0.27^{a}$ |
| $t_c$ | $4.58 \pm 0.39^{a}$ | $4.73 \pm 0.40^{ab}$ | $5.06 \pm 0.42^{b}$ | $5.12 \pm 0.33^{c}$ | $5.23 \pm 0.40^{cd}$ | $5.51 \pm 0.54^{d}$ |

Values are in mean ±SD. At the same row, values with different superscript are significantly different ($P < 0.05$) in means by the DUNCAN's test.

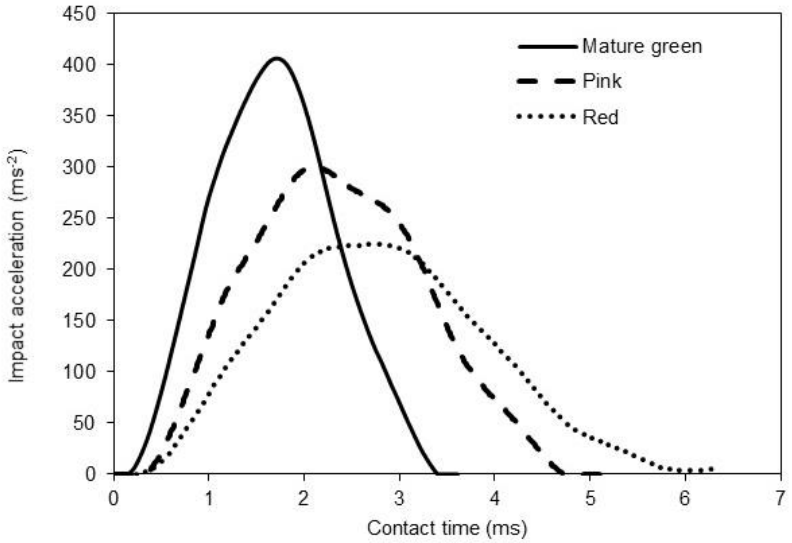


**Figure 3.** Impact acceleration-contact time curves at three maturity stages of tomato fruit.

The range of physical properties and impact parameters for all the fruits tested were given in Table 3. Furthermore, the 10 measured and derived impact parameters used in this study and correlation coefficients between pairs of parameters were shown in the Pearson correlation matrix in Table 4. As seen in Table 4, the correlations between $F_T$ and nondestructive impact parameters were significant at 1% level except $t$ and $(1/t)^{2.5}$.

**Table 3.** Range of physical properties and impact parameters for all the fruits tested

| Measured Variable | Mean | Minimum | Maximum |
|---|---|---|---|
| $m$ (Fruit mass, g) | 103.89 | 78.94 | 162.93 |
| $D_1$ (Equatorial diameter, mm) | 60.49 | 54.38 | 75.09 |
| $H$ (Height, mm) | 48.37 | 42.97 | 70.00 |
| $D_2$ (Thickness, mm) | 59.72 | 51.16 | 73.94 |
| $F_T$ (Tomato firmness, N mm$^{-1}$) | 1.97 | 0.82 | 4.75 |
| $A$ (Maximum acceleration, m s$^{-2}$) | 309.53 | 200.14 | 429.46 |
| $t$ (Time @ A, ms) | 2.12 | 1.71 | 2.94 |
| $t_c$ (Contact time, ms) | 5.12 | 3.83 | 6.66 |
| $A/t$ (m s$^{-3}$) | 148.03 | 31.20 | 68.08 |
| $A/t_c$ (m s$^{-3}$) | 61.94 | 30.05 | 112.20 |
| $A/t^2$ (m s$^{-4}$) | 71.30 | 23.15 | 146.44 |
| $A/t_c^2$ (m s$^{-4}$) | 12.52 | 4.51 | 29.32 |
| $(1/t)^{2.5}$ (s$^{-2.5}$) | 0.16 | 0.07 | 0.26 |
| $(A/t)^{1.25}$ m$^{1.25}$ s$^{-3.75}$ | 519.91 | 195.54 | 997.98 |
| $A^{2.5}$ (m$^{2.5}$ s$^{-5}$) | 1,757,467.23 | 566,689.39 | 3,822,241.05 |

**Table 4.** Correlation coefficients (R) between tomato firmness ($F_T$) and nondestructive impact parameters

| Parameters | $A$ | $t$ | $t_c$ | $A/t$ | $A/t_c$ | $A/t^2$ | $A/t_c^2$ | $(1/t)^{2.5}$ | $(A/t)^{1.25}A^{2.5}$ |
|---|---|---|---|---|---|---|---|---|---|
| $F_T$ | 0.87** | -0.32$^{ns}$ | -0.66** | 0.74** | -0.86** | 0.65** | 0.85** | 0.32$^{ns}$ | 0.75** | 0.92** |

**: significant at 0.01 level, ns: non-significant

The use of the 8 impact measurement parameters that was reduced by Pearson correlation matrix can be complicated in real-time application in concerning with numerical and logical processing. Therefore, to reduce the number of impact parameters, stepwise regression analysis was used to find out the most significant parameters in firmness assessment. The stepwise regression analysis showed that the $A/t$, $A/t_c^2$ and $A^{2.5}$ were the three most dominant parameters with analytical results given in Table 5.

**Table 5.** Statistical results of the dominant impact parameters according to stepwise regression analysis

| Parameters | Beta | t | F value | Prob.>F |
|---|---|---|---|---|
| Constant | | 3.774 | 165.441 | 0.000 |
| $A^{2.5}$ | 1.154 | 8.103 | | 0.000 |
| $A/t$ | -0.837 | -4.861 | | 0.000 |
| $A/t_c^2$ | 0.586 | 2.906 | | 0.006 |

After the dominant impact parameters were selected according to stepwise regression analysis, multiple linear regression (MLR) was used to determine the linear relationship of selected parameters to tomato firmness. In order to search for the best relationship for predicting $F_T$, three models were fitted to the data using multiple linear regression (MLR). The best among them, based on standard error of calibration (SEC), standard error of prediction (SEV), multiple regression coefficients $R^2$ and descriptors in the model was selected. The performance of the calibration models for prediction of $F_T$ was tested with validation set. The fruits were randomly segregated into calibration and validation sets, with 52 tomatoes in the calibration set and 36 tomatoes in the

validation set. Table 6 shows the results of multiple regression with calibration set of 52 tomatoes when one, two or three of these variables were used in the analysis.

**Table 6.** Multiple regression models for predicting tomato firmness based only on parameters obtained from the stepwise regression using calibration data sets

| | Model: $F_T = \beta_0 + \beta_1 x A^{2.5} + \beta_2 x A/t + \beta_{3x} A/t_c^2$ | | | | |
|---|---|---|---|---|---|
| No. of parameters used | $\beta_0$ | $\beta_1$ | $\beta_2$ | $\beta_3$ | $R^2$ |
| 1 | -0.34 | $1.33 \times 10^{-6}$ | | | 0.86 |
| 2 | 0.759 | $1.97 \times 10^{-6}$ | -0.015 | | 0.89 |
| 3 | 1.41 | $1.65 \times 10^{-6}$ | -0.026 | 0.127 | 0.91 |

A multiple linear regression model which includes $A^{2.5}$, $A/t$ and $A/t_c^2$ could predict tomato firmness with a coefficient of multiple determination ($R^2$) and the standard error of calibration (SEC) values of 0.91 and 0.28 N mm$^{-1}$, respectively. In the case of using the validation data set, $R^2$ and standard error of validation (SEP) were found to be 0.92 and 0.28 N mm$^{-1}$, respectively. A comparison of scatter plots of measured $F_T$ values versus computed one for calibration (Fig. 4) and validation (Fig. 5) sets of samples also showed that the measured and predicted tomato firmness values gave very close results. Results showed that a strong and statistically significant improvement in model performance was observed when three impact parameters were used to predict the tomato firmness for both calibration and validation data set. Therefore, the developed MLR model using impact parameters $A^{2.5}$, $A/t$ and $A/t_c^2$ thus may be able to determine the tomato firmness of greenhouse tomato for harvest and post-harvest assessments.

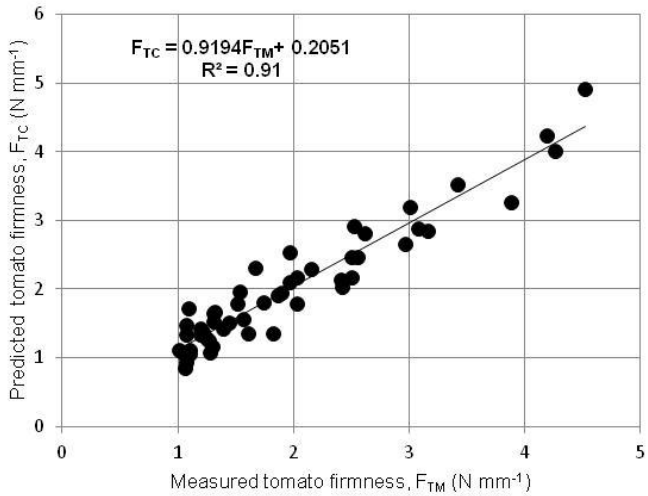


**Figure 4.** Measured versus predicted tomato firmness of calibration sets of samples.

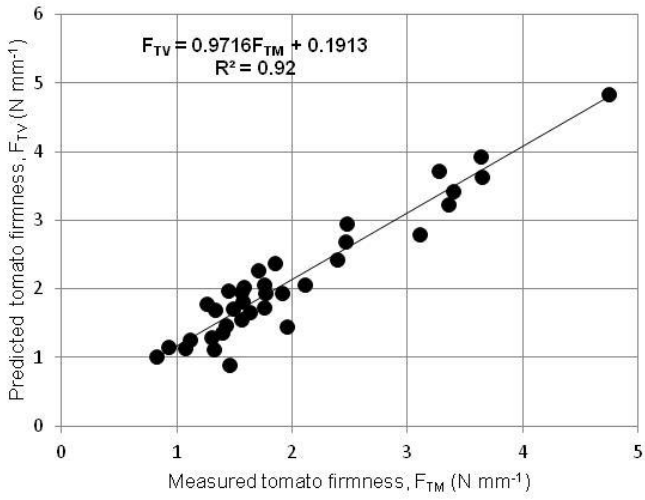


**Figure 5.** Measured versus predicted tomato firmness of validation sets of samples.

## CONCLUSIONS

*Bandita F1* greenhouse tomatoes were tested in laboratory conditions by a low-mass impact device to evaluate nondestructively the firmness of tomato. By multiple linear regression (MLR), precise calibration model could be obtained. Precision of the developed model was proved by the validation data sets. A linear model based on three impact parameters extracted by the stepwise regression analysis can predict tomato firmness ($F_T$) with a coefficient of multiple regression coefficient ($R^2$) and standard error of the calibration (SEC) of 0.91 and 0.28 N mm$^{-1}$ for calibration data sets. The performance of the calibration model for prediction of $F_T$ showed similar results with validation data sets.

Although this study focuses on the firmness assessment of greenhouse tomatoes, further research based on MLR method is needed in order to develop a more accurate models for prediction of tomato firmness nondestructively by using a wider number of parameters for low-mass lateral impact device.

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Frey, R. 1958. Zur Kenntnis der Diptera brachycera p.p. der Kapverdischen Inseln. *Commentat.Biol.* **18**(4), 1–61.

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- 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
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- Use 'kg ha$^{-1}$' (not 'kg/ha');
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