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Resource assessment for potato biorefinery: Side stream potential in Northern Ostrobothnia

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Abstract. Potato industry side-streams consist of a significant amount of the original biomass. However, tightened demands of EU legislation together with the costs of side stream processing have forced potato industry towards more efficient use of the raw material. For this purpose, we have examined the possibility to recover main fractions from potato side streams, such as proteins, fibers and starch, and utilize them in a manner of biorefinery concept. The aim of the present research was to evaluate the potential for a potato biorefinery based on biomasses available at area of Northern Ostrobothnia, Finland. Study shows, that there is enough side-streams available to build a concept, which produces more value added products, like fibers and proteins. In this report, the main conclusions of the research are presented together with state-of-art on potato waste water processing technologies and current applications of their products.

Key words: side streams, potato, biorefinery, starch, fiber, protein, resource assessment.

INTRODUCTION

Potato production can be conducted in a variety of different conditions, which makes it a commonly cultivated crop across the world, also grown in all EU countries. Global annual production of potato is over 350 million tons from which EU countries produce nearly 55 million tons (FAO, statistics from 2012). Finnish annual production is approximately 500,000 t corresponding to harvested area of 20,700 ha.

The geographical scope of the table potato market is rather wide; competition between production areas is enabled due to tolerance of potatoes to carriage and thus low transportation costs. The rising demand for potato products in Central and Eastern Europe, in Asia and South America also offers new business opportunity for European potato producers. Potatoes used for processing have lower unit value, which leads to increased transportation costs compared to table potatoes. Thus the processing plants are located close to the cultivation areas.

The main center of production for Finnish potatoes is in Ostrobothnia, where the conditions are most suitable for the purpose. The present research focuses on area of

Northern Ostrobothnia, which extends across Finland from the Gulf of Bothnia coast to the Russian border and has an area of 37,000 km² and population of about 380,000 people (7.2% of the Finnish population). The harvest area of potato in the region is approximately 3,800 ha accounting to 114.9 million tons (Tike, 2013). The majority of harvest is used as seed potato or as food (table potato or food industry). Starch potato is not cultivated in large extend.

Potato cultivation and processing creates significant amounts of side streams that are not included in the main products. These are especially produced during processing of potato, *i.e.* peeling, packaging and washing, while harvesting residues comprise smaller but still remarkable share of non-used biomass. The amount of produced side streams varies from process to process; in some peeling processes up to half of the biomass ends up to side stream (Peusa & Piilo, 2006). Further processing of side streams is costly and thus affects economy of industry. It is also notable that the biological burden of these side streams is relatively high. For instance, biological oxygen consumption of potato peel mass can be 10,000 mg L – 1 (Peusa & Piilo, 2006) while that of municipal waste is only approximately 200 mg L – 1 (Riekkinen, 2007). Additional pressure comes from EU directives regarding end-of waste criteria for biodegradable waste subject to biological treatment (compost/digestate) in accordance with Article 6 of Directive 2008/98/EC of the European Parliament and of the Waste Framework Directive (JRC 2014). Thus, it is obvious that the valorization of potato industry side streams would be beneficial for both environment and industry.

Data on produced side streams in the research area were collected by interviewing of industry, supermarkets and potato farmers. Based on the survey, preliminary feasibility evaluation was prepared for potato biorefinery concept around main compounds in the side streams. By definition biorefining means sustainable processing of biomass into spectrum of marketable products and energy. The basis of this research was the assumption that biorefinery is the most feasible manner for optimal use of the produced side streams. The preliminary biorefinery concept was thus build based on the known composition of potato, the composition of processing side streams and the estimation of resource potential and location. In our further research we intend to develop necessary separation and purification processes for these compounds, and to integrate the developed processes as potato biorefinery concept.

MATERIALS AND METHODS

An assessment of potato industry side streams in Northern Ostrobothnia was conducted by direct industry survey. The survey was conducted during January–February 2012. The target group included 28 packing plants (seed and food potatoes), 7 potato processing plants (peeling plants, refining plants), and 11 large retail stores (supermarkets). The target companies were asked to describe the amount of produced side streams from potato production, processing and distribution, and the current use of these streams. The companies were also asked the current costs of processing the side streams and to evaluate, which price they would sell side stream potatoes. In total the survey included 15 questions.

Resource estimates were made of the quantities of materials produced and the potential for their use in biorefinery. Quantities of materials were estimated by summing the answers from industry survey. Based on known compositions of potato

industry side streams, the initial biorefinery plan included separation and upgrading of starch, fibers, proteins and solid residues.

The main compound in potato is water, forming approximately 63–87% w/w (Storey, 2007, Burlingame et al., 2009). The other main compounds are starch (9.1– 22.6%) and other carbohydrates (mainly glucose, fructose and saccharose); and fibers, from which 0.87–1.22% are soluble and 0.41–2.53% insoluble. Potato bulb contains small amounts of proteins (0.85–4.2%), fat (0.05–0.51%), trace elements and vitamins (6.5–34 mg 100 g^{-1} of vitamin C). In addition, potato contains toxic thermo tolerant glycoalkaloids 0.071–175 mg 100 g^{-1} (Burlingame et al., 2009).

Potato starch is located in starch granules composed of amylose and amylopectin (Storey 2007). Starch concentration depends on species, cultivation conditions and season; in spring potato it is 10% and in starch potato 21% (Kangas et al., 2007). The concentration of starch is highest in peels and smallest in the middle of potato in which it can be even totally absent (Karlsson & Eliasson, 2003; Virtanen, et al., 2005). Native potato starch is most suitable as filler and 'binding agent' and thus its properties have been changed to allow wider spectrum of applications, first with chemical means (Kraak, 1993) and later also biologically (Jobling, 2004). Modified starches are used e.g. in building and textile industries (Lyckeby, 2012). Starch production creates potato fruit juice (PFJ) and potato pulp as side streams, both of which contain proteins and fibers. These side streams could be utilized better which would eventually also improve the economy of industry.

Potato fiber includes other carbohydrates than starch, i.e. cellulose, hemicellulose, pectic substances and pentosan. Those are present in tuber cell walls and intracellular structures, and they comprise approximately 2.3% w/w of potato (Storey, 2007). Main polysaccharides in fibers are cellulose (10–12%), pectin (0.7–1.5%) and hemicellulose (1%). Pectic substances are mainly protopectin (70%), soluble pectin (10%) and pectic acid (13.3%). Potato hemicellulose is composed of glucuronic acid, xylose, galacturonic acid and arabinose (Kadam et al., 1991). Additionally starch resistant to small intestine digestion is present.

Potato contains 6.9–46.3 g protein per kg of wet weight, the concentration being highest in peel layer (Karlsson & Eliasson, 2003). The quality of potato protein depends on amino acid composition. The quality is measured using biological value, which describes the proportion of nitrogen in protein available for human consumption. If all of the nitrogen can be utilized in human body the biological value is 100, which is the reference value from egg white. Potato protein has biological value of 90–100 which means excellent composition of amino acids for human nutrition. It contains nearly all essential amino acids for human with only exceptions of methionine and cysteine (Storey, 2007). In addition, potato waste may include also high-valued protein fractions that could be utilized commercially (Schieber & Saldaña, 2009).

Minerals comprise 1.1% of potato tuber weight (Storey, 2007). According to research by MTT (Ahokas et al., 2012) dry matter mean value is 22.3%. The average concentrations of phosphorus, potassium, calcium, sulfur, sodium, magnesium, copper, mangane, zinc, iron and boron of five different cultivars has been studied in field tests during 2005–2011 (Table 1).

Table 1. Minerals in potato tubers (Ahokas et al., 2012)

Element g kg^{-1}	P	K	Ca	S	Na	Mg	Cu	Mn	Zn	Fe	B
	1.9	21.7	0.3	1.2	1.5	0.0	0.0046	0.0076	0.0113	0.0339	0.0065

The majority of potato industry side streams are formed during peeling, cutting and packaging. Peeling of potatoes produces washing waters that include peel residues and PFJ. In wet peeling 25–50% of the raw material ends up into residues. Its solid content is 10–15%, which includes also some earth. Therefore, it is not usable as animal feed. Dry peeling produces 50–100% less side streams than wet peeling. The produced side streams include earthy water (produced during washing), peel mass (pure potato), starch and PFJ. Peel mass (potato pulp) resulting from the industrial starch processing is highly viscous and contains 16–17% by weight of dry matter of which 30–35% starch and 60–65% non-starch polysaccharide material (NSP) (Mayer et al., 2008). Additionally minerals are included (K : 20 g kg^{-1}, C : < 0.8 g kg^{-1}, P : 1.4 g kg^{-1}, Mg : 0.85 g kg^{-1}). Peel mass may also be stored for some days due to natural lactic acid fermentation; however, it is advisable to use it fresh (Peusa & Piilo, 2006). The energy content of peel mass is 13.6 MJ Kg^{-1} (Peusa & Piilo, 2006).

Table 2. The yield estimate of value added products from potato residue feedstock.

Product	Yield kg t^{-1}	Available products in market
Recovered wastewater	2,000	Process Water,
Ethanol	33[1]	Several potable vodka brands
Potato protein	10[2]	Tubermine®, PRO GO™,
Potato protein isolate	0.6[3]	Slendestra™, SUPRX™, Gly-Sea-Max™, Solathin™
Potato fiber	38[4]	Vitacel™, PENFibe®RO, Pofiber (Semper), Potex

[1]Izmirlioglu & Demirci (2012) (33g L-1 ethanol from waste potato mash); [2]Approximate value based on Karup Kartoffelmelfabrik (2007); [3]US Patent 6414124 2002 (900 mg kg^{-1} potato); [4]Mayer et al. (2012) (24% by weight of the pulp dry matter)

The amount of produced PFJ is approximately 70% the whole peel mass. It is easily spoiling and difficult to handle and its biological oxygen consumption is rather high. PFJ contains 23.5% solids which is formed by starch (17.4%), proteins (2.5%), fibers (1.8%), soluble carbohydrates (0.5%) and minerals (1%) (Bergthaller et al., 1999). Nutrients are present as follows: N : 0.33% (from which half is soluble), P : 0.045%, K : 0.47%, Mg : 0.03% and Ca : 0.002% (Riekkinen, 2007). PFJ may be utilized as fertilize in farming according to respective legislation (Peusa & Piilo, 2006).

Cutting processes produce classification and cutting residues. In addition, spoiled, under – or over dimensioned and incorrectly shaped potatoes are discarded to waste during packaging (Peusa & Piilo, 2006). The amount and solid content of side streams vary between processes.

The preliminary biorefinery concept was designed as a basis for the resource assessment and estimation of biorefining potential in the region. This concept is based on literature review on potato side streams, composition of potato and existing technologies for separation and purification of main components starch, fibers and

proteins. To calculate material balance, certain yield values were chosen based on the literature review. These yield values are presented in Table 2.

RESULTS AND DISCUSSION

The response rate of industry survey was 72%, which was considered sufficient to assessment of resource potentials. The results of industry survey are summarized in Table 3.

Table 3. The results of industry survey. Standard error of the mean in parentheses

Product	Answers (n)	Treatment capasity T a^{-1}	Sorting waste t a^{-1}	Potato residue (in total) t a^{-1}
Seed potato packing	14	450–9,000 (1,513)	25–250 (44)	1,600
Food potato packing	8	1,500–10,000 (955)	250–1,000 (95)	3,700
Potato peeling	4	180–3,500 (706)	90–480 (80)	1,100
Markets	7		8–90 (10)	220

Packaging plants classify the potatoes in order to remove spoiled, under - or over dimensioned and incorrectly shaped potatoes. A minor part of harvest is usually left unsold e.g. due to excess supply. The annual processing volumes at seed potato packaging plants and food potato packaging plants were 450–9,000 t a^{-1} and 1,500–10,000 t a^{-1}, respectively (Table 3). Potato processing steps and produced side streams at a typical seed and/or food potato packaging plant are presented in Fig. 1.

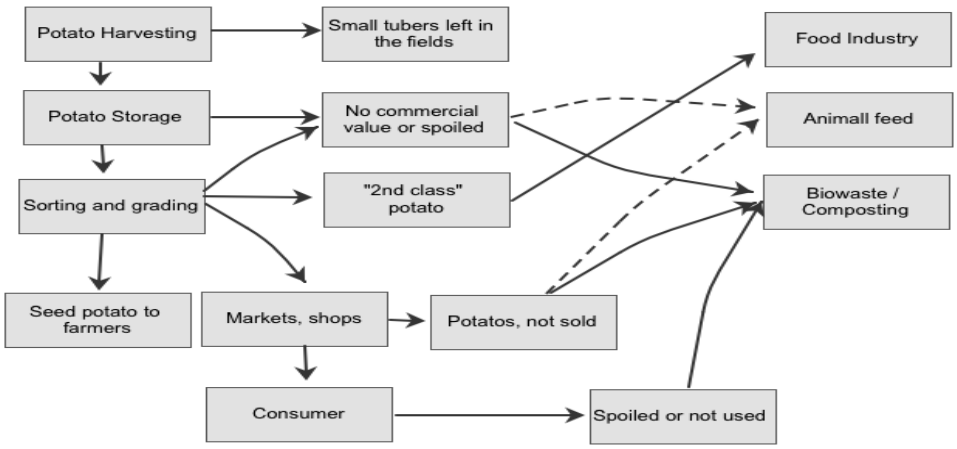

Figure 1. Process steps and produced side streams at a typical seed or food potato packaging plant. Solid line – primary use of side stream, dashed line – secondary use of side stream.

The amount of unsold potatoes at seed and food potato plants are 3–15% and 10–17% from total raw material, respectively. The difference is due to possibility of seed potato packaging plants to direct unsold potatoes to be used as food. In total, side

streams from potato packaging plants count to approximately 5,000 t a^{-1}, from which approximately 85% is further utilized at food production (2^{nd} class potatoes). Thus, only 15% of packaging plant side streams, accounting to 1,100 t a^{-1}, can be included in the total side stream potential of the region. This residue is currently composted and used either as fertilizer or animal feed. However, companies are willing to sell their side streams if the income from it would be higher than current prices for 2^{nd} class potatoes, and the transportation of biomass would be arranged without extra cost. Based on our estimation, the price should be approximately 13 euro t^{-1} without transportation cost.

Approximately half of the interviewed packaging plants are able to wash the potatoes before packaging. Washing waters contain mostly earth and they are processed by sedimentation and sand filters. The recovery of biomass compounds from these waters can be considered unprofitable (Lehto et al., 2007).

Based on visual evaluation of potato farmers, approximately 5% of the harvest is left to fields. The majority of this residue is formed by under dimensioned potatoes. The exact amount of the residues is likely to depend on several factors, such as earth humidity and specifications of harvest equipment. The farmers recognized possibilities for improved harvest e.g. via minor equipment adjustments; however, this choice is not attractive unless the price for 2^{nd} class potatoes increases. In regional context the harvest residues may be up to 6,000 t a^{-1} accounting a significant amount of biomass.

The major potato processing plants in the region are chip production plant in Pyhäntä and multiproduct plant in Vihanti. In these processes 27–54% of the raw material ends up to side streams which accounts to 16,100 t a^{-1}. Vihanti plant share of this is 15,000 t a^{-1}, from which the majority is used in animal feed. This side stream is composed of classification and peeling residues of 1^{st} class potatoes, and peeling residues of 2^{nd} class potatoes that are used for potato flake production.

Other processing plants, mostly operating as peeling and cutting units, direct their peeling biomasses either as feed or composting. The estimated total side stream potential from peeling plants is 1,100 t a^{-1}. The process steps of potato processing plants are presented in Fig. 2.

Peeling plants that employ grinding method produce large amounts of starch containing water and PFJ. According to survey this side stream can be of similar size as the solid peeling residue. The surveyed companies used sedimentation, filtration and centrifugation for separation of solids. Centrifuges were generally considered effective but also too expensive for small companies.

Answers were received from 7 out of 11 surveyed retail stores. The annual amount of unsold potatoes, vegetables and fruits was 8–90 t a^{-1} in each store. The majority of this residue consisted of fruits and vegetables. Thus, the potential of potato side stream from the retail stores is rather low. Furthermore, retail stores suggested that the side streams should be removed in short sequences, several times a week, which together with small amounts of produced side streams would lead to relatively high logistic cost. Currently these residues are mostly composted which also generates costs to the companies. Small portion of the residues ends up as animal feed.

In terms of feedstock potential the most feasible location for the biorefinery would be in the proximity of Vihanti plant. The biorefinery would operate using side streams of potato flake production and peeling residues which results to minimal effects on material balances in plant itself. This would reduce the use of side streams

for animal feed production. However, if bioethanol is produced in the biorefinery, also 2nd-class potatoes could be applied for this purpose.

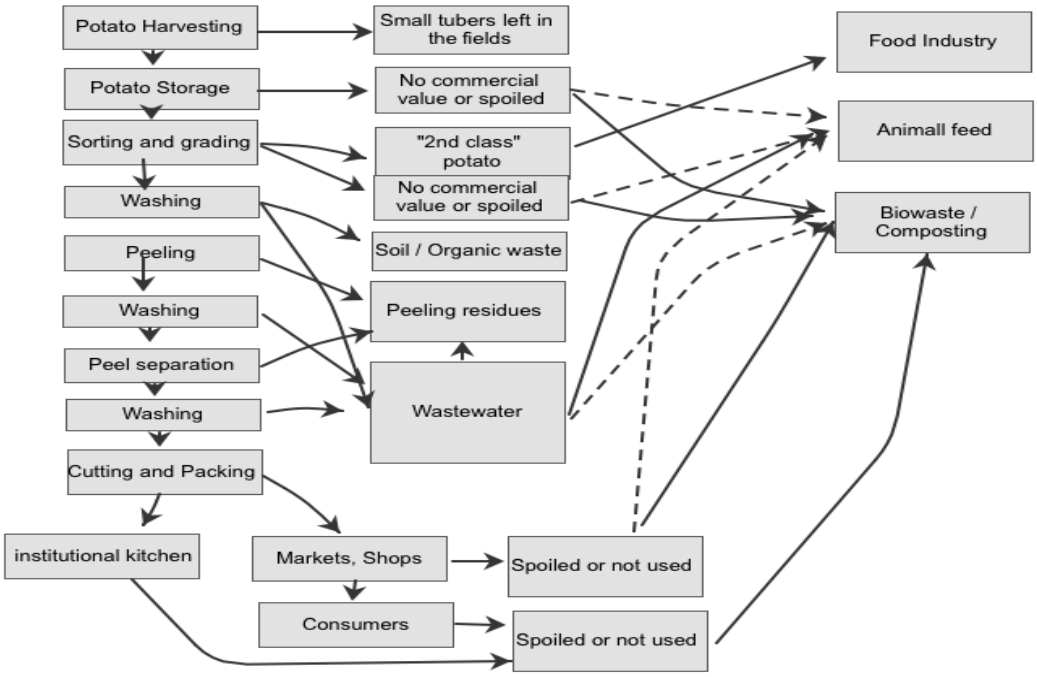

Figure 2. Process steps and produced side streams at potato processing plants. Solid line – primary use of side stream, dashed line – secondary use of side stream.

Based on rough estimations, the capacity of biorefinery could be approximately 20,000 t a^{-1} biomass feedstock for its operation. According to the present results 17,000 t a^{-1} could be acquired within the studied region with relatively small arrangements. The acquisition of retail store side streams could add to regional side stream potential if it could be feasibly arranged. The potential could be further increased if the use of harvest residues could be arranged in a feasible manner.

Considering maximal logistic cost of 10 euro t^{-1}, feasible range for feedstock transportation would be approximately 130 km (Paappanen et al., 2008). The respective zone of supply would include the whole region of Northern Ostrobothnia and areas of Central Ostrobothnia. It can be assumed that sufficient amount of feedstock is available within this area. However, it is notable that the availability of side streams depends on both annual harvest and potato demand, and therefore the annual feedstock demand of 20,000 t may not be available at all times.

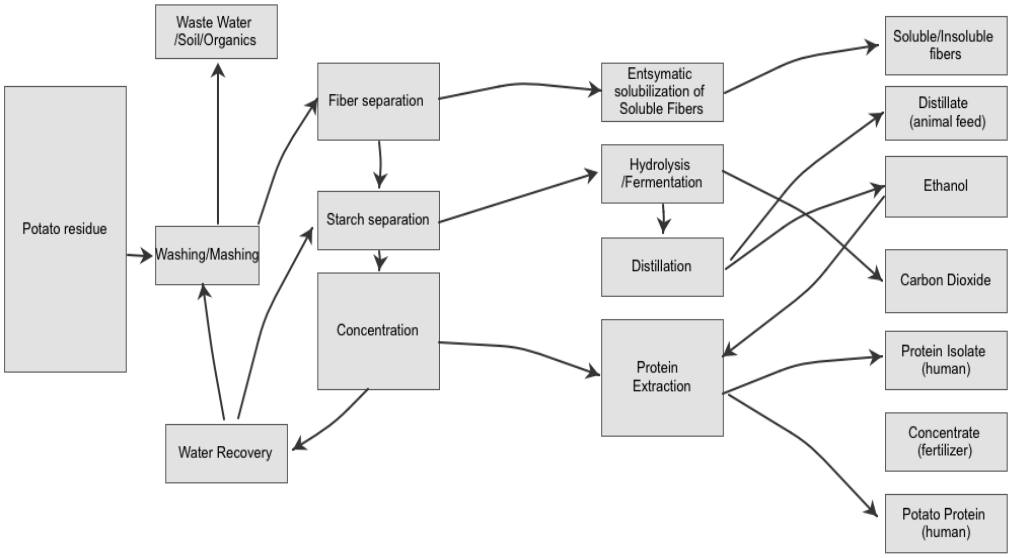

Figure 3. Estimation of material flow for potato biorefinery.

Material flow was estimated using the values from literature presented in Table 2. Assuming a feedstock of 20,000 t a^{-1} the potato biorefinery would produce 660 t a^{-1} ethanol, 760 t a^{-1} potato fiber, 200 t a^{-1} potato protein and 12 t a^{-1} protein isolates Also 40,000 tons of waste water can be recycled. Estimation of material flow is presented in Fig. 3.

CONCLUSION

1. The total potato side stream in the studied region would be about 17,200 t a^{-1}. The biggest side stream (15,000 t a^{-1}) comes from Vihanti potato product plant. At the present, main part (85%) of packing plants side stream is transported to Vihanti plant for potato flake production.
2. Only 15% of packing plants side stream (1,100 t) and the peels (1,100 t) can be added to Vihanti plant side stream, when estimating total side stream of the region.
3. Based on rough estimations biorefinery would require approximately 20,000 t a^{-1} biomass feedstock for its operation. According to the present results 17,000 t a^{-1} could be acquired within the studied region with relatively small arrangements.
4. The acquisition of retail store side streams could add to regional side stream potential if it could be feasibly arranged.
5. The potential could be further increased if the use of harvest residues could be arranged in a feasible manner.
6. In terms of biorefinery operations, significant role is played by the feedstock availability.
7. It is important to obtain low-cost but high yield chemical recovery process.
8. According to presented biorefinery concept, potato protein conversion, including ethanol recovery process and fiber production to high value products seems to be the most beneficial technical solution.

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REFERENCES

Ahokas, M., Välimaa, A.-L., Kankaala, A., Lötjönen, T. & Virtanen, E. 2012. Perunan ja juuresten sivuvirtojen arvokomponenttien hyötykäyttö. MTT reports 67. p.48. ISBN: 978-952-487-410-6. *Available at*: http://www.mtt.fi/mttraportti/pdf/mttraportti67.pdf

Bergthaller, W., Witt, W. & Gpldau, H.-P. 1999. Potato Starch Technology. *Starch/Stärke* **51**, 235–242.

Burlingame, B., Mouillé, B. & Charrondière, R. 2009. Nutrients, bioactive non-nutrients and anti-nutrients in potatoes. *J. Food Comp. Anal.* **22**, 494–502.

European Union. Comission directive 2008/100/ EC. Official Journal of the European Union. 2008; L285:9–12. Referred 12.4.2012. *Available at*: http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:285:0009:0012:EN:PD

FAO statistics from 2012. Referred 13.11.2013. *Available at*: http://www.fao.org/statistics/data bases/en/.

Gulten Izmirlioglu, G. & Demirci, A. 2012. Ethanol Production from Waste Potato Mash by Using *Saccharomyces Cerevisiae*. *Appl.Sci.* **2**, 738–753.

Jobling, S. 2004. Improving starch for food and industrial applications. *Curr. Opin. Plant Biol.* **7**, 210–218.

JRC SCIENTIFIC AND POLICY REPORTS 2014. End-of-waste criteria for biodegradable waste subjected to biological treatment (compost & digestate): Technical proposals. Referred 12.1.2014. *Available at*: http://ftp.jrc.es/EURdoc/JRC87124.pdf.

Kadam, S.S., Dhumal S.S. & Jambhale, N.D. 1991. Structure, nutritional composition and quality. In Salunkhe, D.K., Kadam, S.S., Jadhav, S.J. (eds.): *Potato: production, processing and products*. Boca Raton, Florida, pp. 9–35.

Kangas, A., Laine, A., Niskanen, M., Salo, Y., Vuorinen, M., Jauhiainen, L. & Nikander, H. 2007. Results of official variety trials 2000–2007. MTT reports 150. p. 202. ISBN-978-952-487-147-1.

Karlsson, M.E. & Eliasson A.-C. 2003. Gelatinization and retrogradation of potato (*Solanum Tuberosum*) starch in situ as assessed by differential scanning calorimetry (DSC). Lebensm.-Wiss. u.-technol. 36, pp. 735–741.

Karup Kartoffelmelfabrik Denmark 2007. Novel Potato Protein. New innovative protein proces s. Referred 30.3.2012. *Available at*: http://www.newpotatopro.dk/brochure_final.pdf

Kraak, A. 1993. Industrial applications of potato starch products. *Ind. Crop. Prod.* **7**,107–112.

Lehto, M., Salo, T., Sorvala, S., Kemppainen, R., Vanhala, P., Sipilä, I. & Puumala, M. 2007. [Wastes and wastewaters from potato and vegetable peeling processes]. Maa- ja elintarviketalous 94: 77 s. Referred: 30.3.2012. Available at: http://www.mtt.fi/met/pdf/met94.pdf.

Lyckeby Stärkelse 2012. Referred 3.4.2012. *Available at*: http://epi.lyckeby-industrial.com/LyckebyTemplates/Page.aspx?id=1948

Meyer, A.S., Dam, B.P. & Lærke, H.N. 2009. Enzymatic solubilization of a pectinaceous dietary fiber fraction from potato pulp: Optimization of the fiber extraction process. *Biochem Eng J.* **43**(1), 106–112.

Paappanen, T., Lindh, T., Kärki. J., Impola, R., Taipale, R., Leino, T., Rinne, S., Lötjönen, T. & Kirkkari, A-M. 2008. [Development of reed canary grass fuel chain]. VTT Research notes 2452. Referred 30.3.2012. *Available at*: http://www.vtt.fi/inf/pdf/tiedotteet/2008/T2 452.pdf.

Peusa, J. & Piilo, T. 2/2006. Perunat ja vihannekset kuorinta- ja paloitteluprosessissa. Referred 5.4.2012.

Schieber, A. & Saldaña, M. 2009. Potato peels: A source of nutritionally and pharmacologically interesting compounds- a review. *Glo. Sci. Books, Food*. **3**, 23–29.

Storey, M. 2007. The harvested crop. In: Vreugdenhill, D. (ed.) *Potato Biology and Biotechnology, Advances and Perspectives*. Langford Lane, Oxford, pp. 441–470.

Tike, 2013. Statistics of Information Centre of the Ministry of Agriculture and Forestry, Finland: Utilized agriculture area. Referred 22.01.2014. *Available at*: http://www.maataloustilastot.fi/en/utilised-agricultural-area-2013-regional-preliminary-data_en

US PATENT 6414124 B1 2002 Methods for the isolation of proteinase inhibitor proteins from potato tubers. Referred 30.4.2012. *Available at*: http://www.google.nl/patents/US6414124

Virtanen, E., Tuikkanen, N. & Hohtola, A. 2005. The effects of the potato cultivars and the cultivation techniques on the potato starch accumulation, localization and starch content in potato tubers in 2004-2006. In Ritter, E., Carrascal, A. (eds.): *16th triennial conference of the EAPR European Association for Potato Research.* Bilbao, Spain. pp. 898–899.

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Effect of cold treatment on the amino acid composition of veal

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Abstract. Veal is a promising raw material for use in the daily diet, as well as for production of functional and dietary foods. However the effect of cold treatment on the amino acid composition of veal has not been sufficiently studied. The aim of this study was the amino acid composition analysis of veal subjected to various variants of cold treatment.

The selected material under research was muscle tissue of hip parts from calves, grown in the Leningrad Region, Russia and aged no more than 3 months. Cooling to 4 ± 1°C and rapid freezing to the temperature of minus 18°C at the cooling air temperatures of minus 24°C and minus 35°C were used as variants of cold treatment. Amino acid composition analyses were carried out using precolumn derivatization with phenylisothiocyanate and reversed-phase gradient HPLC on the Shimadzu 20-AD chromatograph with spectrophotometric detection at 254 nm.

The results show the effect of cold treatment on the content of free amino acids and total amino acid composition of veal. In many respects changes in amino acid composition are concerned with moisture losses during the refrigerating treatment. The dependence between the change in amino acid content and the structure of its side chain group type is shown. Amino acid score for essential amino acids was calculated and conclusions about changes in biological value of veal protein were made.

The obtained data can be used in biological value calculation of the multi-component products and food rations with veal subjected to refrigerating treatment.

Key words: cooling, freezing, HPLC, protein, meat.

INTRODUCTION

The main sources of protein for humans are products of animal origin and some legumes. Protein biological value depends strongly on the type of raw material, and it determines formation of the daily diet and reasoning of the consumption norms for animal and vegetable proteins. The physiological average daily requirements in proteins are systematically studied and analyzed in the decisions of the FAO/WHO and national organizations of different countries, including the Russian Federation (FAO 1970; FAO/WHO 1991, 2007, 2011). However the effect of cold treatment on the amino acid composition of meat has not been sufficiently studied.

Because of its chemical composition, veal is a promising raw material for use in the daily diet, as well as for production of functional and dietary foods. Some of these products are characterized by high protein content, at that the share of complete proteins should be at least 60% of the total protein content. Furthermore, the internal organs of calves are used to obtain a large number of biologically active substances, it

is necessary to store and rationally process the rest of the carcass. In this regard, a study of veal is of particular interest.

In chicken muscle proteins after freezing at -30°C and frozen storage at -5°C for 10 weeks, the changes occurring in myofibrillar proteins as a result of frozen storage were indicated by the loss of -SH groups, ATPase activity, solubility, and water-holding capacity (Khan et al., 1968). Also the results show that rapid freezing preserves the integrity of muscle proteins to a greater extent than slow freezing (Khan & Berg, 1967). Freezing of bovine muscle has a denaturating effect on myofibrillar proteins; the lower the freezing rate the greater the loss (Wagner & Anon, 1985).

Disulfide bond formation with the concomitant decrease in sulfhydryl group was found in all species of some tropical fish during frozen storage (Benjakul et al., 2003). It was found that the denaturation of proteins during freezing is closely related to surface-induced denaturation (Chang et al., 1996). The review was made to describe the various stages of freezing of freeze-dried therapeutic proteins and examine the consequences of the various stresses developing during freezing on protein stability; however, the study of changes of amino acid composition was not carried out (Bhatnagar et al., 2007). Decrease in the content of each of sixteen amino acids was found in *Lactobacillus bulgaricus* after freeze-drying (Mitić, 1976).

For other food products effect of freezing to -25°C on amino acid composition was studied for selected species of edible mushroom (Bernaś & Jaworska, 2012). After freezing at temperature of cooling air of -35°C and frozen storage mushrooms contained significantly higher levels (3–118% on average) of leucine, lysine, methionine, and phenylalanine, but lower levels (8–61% on average) of cysteine, histidine, isoleucine, tyrosine and valine, compared with canned products.

Nutritional composition of veal is studied in several works, including the amino acid composition of preruminant calves and special fed veal ribeyes (Williams, 1978; Riss et al., 1983; Williams, 2007). However, information about the changes in amino acid composition of veal depending on conditions of cold treatment could not be found.

The aim of this study was the amino acid composition analysis of veal subjected to different variants of cold treatment.

MATERIALS AND METHODS

The selected material under research was muscle tissue of hip parts from calves, grown in the Leningrad Region, Russia and aged no more than 3 months. The meat was placed in a refrigerator at a temperature of 4 ± 1°C in 2 h after slaughter. After three days of refrigerated storage, the samples with a thickness of 2.0 ± 0.3 cm and a weight of 200 ± 5 g were isolated from the *musculi biceps femoris et gluteus superficialis* and subjected to rapid freezing to -18°C. Freezing at the temperatures of the cooling air of -24°C and -35°C were used as variants of cold treatment. Storage of frozen meat at -18°C held no longer than 24 h. Prior to analysis, each sample was placed in an individual plastic container and defrosted for 4 h at 5°C under natural convection.

Standard samples of amino acids (Sigma), phenylisothiocyanate (Sigma), acetonitrile (HPLC-grade, J.T.Baker) were used for the research. Isopropyl alcohol (HPLC-grade), sodium acetate (*puriss.*), hydrochloric acid (*puriss.*), sodium hydroxide

(*puriss. spec.)* were from Vekton, Russia. Water for analysis was produced with a Milli-Q purification system (Millipore) from twice-distilled water.

Amino acid composition analyses were carried out using precolumn derivatization with phenylisothiocyanate and reversed-phase gradient HPLC. The method is a combination and modification of methods described in Heinrikson & Meredith (1984), Gunawan et al. (1990), Fierabracci et al. (1991), González-Castro et al. (1997). The used method is certified by All-Russian Research Institute of Metrology and has a number of M-02-902-142-07.

Analyses of amino acids derivatives were performed by liquid chromatograph Shimadzu LC-20 Prominence with spectrophotometric detector (254 nm); column with a reverse phase C18 (250 x 4.6 mm, 5 μm, manufactured by Supelco) and a corresponding precolumn; mobile phase – a mixture of 6 mM sodium acetate, pH = 5.5 (component A), 1% isopropyl alcohol solution in acetonitrile (component B), and 6 mM sodium acetate pH = 4.05 (component C). Chromatographic analyses were carried out in a gradient mode. Mobile phase flow rate was 1.2 ml min^{-1}.

Standard samples of amino acids were dissolved in 1 M hydrochloric acid solution. Aliquots of standard solution of 15, 25, 50, 100 and 150 μl were placed in five test-tubes. Hydrochloric acid was removed from aliquots by drying on a water bath at 60°C in a stream of air through the capillary by suction created by an electric vacuum pump. Sodium hydroxide solution (0.10 ml of 0.15 M) was added to the dried amino acids, mixture was stirred, and then 0.35 ml of phenylisothiocyanate in isopropyl alcohol and 0.05 ml of water were added. The solution was thoroughly stirred and left for 20 min at room temperature, then evaporated to dryness at a temperature of 60°C. The dry residue was dissolved in 1 ml of water. The resulting solution was subjected to chromatographic analysis.

Sample preparation for determining the total contents of amino acids included the acid hydrolysis with 6 M hydrochloric acid at 110°C for 16–18 h. Samples (~ 0.2 g) were placed in vials, that were filled with a solution of hydrochloric acid and hermetically sealed. Before sealing the vapor phase was purged for 3 min with nitrogen to prevent oxidation of amino acids by atmospheric oxygen. After cooling, the hydrolyzates were filtered; aliquots (0.2–0.3 ml) were placed in test-tubes and evaporated to dryness on a water bath at 60°C in a stream of air similar to the standard solutions. Sodium hydroxide solution (0.10 ml of 0.15 M) was added to the dried aliquots, mixture was stirred, and then 0.35 ml of phenylisothiocyanate in isopropyl alcohol and 0.05 ml of water were added. The solution was also thoroughly stirred and left for 20 min at room temperature, then evaporated to dryness at a temperature of 60°C. The dry residue was dissolved in 1 ml of water. The resulting solution was subjected to chromatographic analysis.

For the determination of free amino acids, the meat samples were homogenized; sample (5 g) was placed in a glass cup, filled with 50 ml of ethyl alcohol, mixed with a magnetic stirrer, and small amount of fluid was filtered through a membrane filter. Aliquots (0.2 ml) were collected, placed in vials and evaporated to dryness on a water bath at 60°C in a stream of air. Phenylisothiocyanate derivatisation was then performed; samples were dried and then dissolved in 1 ml of water similar to the standard solutions and the solutions after acid hydrolysis. The obtained samples were subjected to chromatographic analysis.

All experiments were performed with at least three replicates; data was processed by methods of mathematical statistics at theoretical frequency 0.95.

RESULTS AND DISCUSSION

Changes in free amino acids after freezing

Free essential and non-essential amino acids content change after veal freezing process is shown in Table 1. As can be seen from Table 1 the total amount of these acids is increased regardless of the freezing temperature. However, more free essential amino acids are accumulated in the meat frozen at a temperature of -24°C (1.92 times) than at -35°C (1.31 times).

Table 1. The contents of free amino acids in veal

Amino acid	Mass fraction of free amino acids in meat, mg g(protein)$^{-1}$		
	before freezing	after freezing at t, °C	
		-24	-35
Essential			
Histidine	1.47 ± 0.06	2.2 ± 0.2	1.15 ± 0.08
Isoleucine	0.17 ± 0.01	0.3 ± 0.03	0.25 ± 0.02
Leucine	0.31 ± 0.02	0.64 ± 0.04	0.44 ± 0.04
Lysine	0.18 ± 0.01	0.76 ± 0.08	0.63 ± 0.05
Methionine	0.021 ± 0.001	0.042 ± 0.004	0.038 ± 0.002
Cysteine + Cystine	trace amounts	trace amounts	trace amounts
Phenylalanine	0.2 ± 0.02	0.31 ± 0.02	0.24 ± 0.02
Tyrosine	0.19 ± 0.01	0.39 ± 0.03	0.29 ± 0.02
Threonine	0.29 ± 0.02	0.77 ± 0.05	0.59 ± 0.05
Tryptophan	trace amounts	trace amounts	trace amounts
Valine	0.24 ± 0.02	0.49 ± 0.03	0.4 ± 0.03
Total:	3.071	5.902	4.028
Non-essential			
Alanine	1.11 ± 0.08	1.7 ± 0.1	2.4 ± 0.2
Arginine	11.0 ± 0.8	15.2 ± 0.9	11.7 ± 0.6
Serine	0.34 ± 0.02	0.60 ± 0.04	0.41 ± 0.04
Aspartic acid & Asparagine	2.6 ± 0.2	1.9 ± 0.2	1.54 ± 0.08
Glutamic acid & Glutamine	2.4 ± 0.1	1.41 ± 0.08	1.2 ± 0.1
Glycine	0.61 ± 0.06	0.74 ± 0.05	0.76 ± 0.06
Proline	0.17 ± 0.01	0.24 ± 0.02	0.19 ± 0.01
Hydroxyproline	0.037 ± 0.003	0.042 ± 0.003	0.032 ± 0.002
Total:	18.267	21.832	18.232
In total:	21.338	27.734	22.26

The content of free non-essential amino acids changed in a lesser degree. Thus, their content in veal, frozen at -24°C increased 1.20 times and at -35°C remained almost unchanged.

Analysis of the data presented in Table 2 shows that the change in the amount of free amino acids after freezing depends not only on the temperature of the process, but also on the structure of their side chain group type. Content of certain amino acids is reduced. Thus, meat freezing at -24°C and -35°C reduced the amount of aspartic acid and asparagine 1.37 and 1.69 times, glutamic acid and glutamine 1.70 and 2.00 times, respectively. Possibly, this change is due to the fact that the polar amino acid with anionic radicals – aspartic acid and its monoamide glutamine have clearly marked hydrophilic properties. Reactions of these acids deamination with formation of nitrogen-free compounds – glutaric and succinic acids could take place during phase transformation of water into ice and freezing. The histidine content decrease in 1.28 times during veal freezing at -35°C was also noted, which can probably be explained by the reaction of decarboxylation and the formation of nitrogenous compound histamine.

However, the content of most free essential and non-essential amino acids has increased in meat frozen at -24°C and at -35°C. It should be noted that the greatest changes take part in the polar amino acids with hydrophilic nonionogenic and cationic radicals (Table 2). Thus, the amount of polar amino acids with hydroxyl nonionogenic radicals increased 2.15 and 1.57 times in meat frozen at -24°C and -35°C, respectively. The biggest changes from the polar amino acids with cationic radicals underwent lysine, its content significantly increased 4.22 and 3.50 times in meat, frozen at -24°C and -35°C. The amount of arginine in meat frozen at -35°C mostly unchanged.

The freezing temperature has no significant effect on the content of non-polar amino acids with hydrophobic side chains. Thus, the amount of amino acids having aliphatic hydrocarbon radicals increases 1.57 and 1.69 times in meat frozen at -24°C and -35°C respectively. The exceptions are the amino acids alanine, leucine and isoleucine. The amount of alanine increased 1.53 and 2.16 times, valine – 2.04 and 1.67 times, isoleucine 1.76 and 1.47 times in meat frozen at -24°C and -35°C, respectively.

The amount of phenylalanine is increased 1.55 and 1.20 times, methionine – 2.00 and 1.81 times in meat frozen at -24°C and -35°C respectively.

Accumulation (content increase) of free amino acids during meat freezing obviously is due to the proteolysis of muscle and connective tissue proteins. It is known that proteins ability to be attacked by proteolytic enzymes is greatly enhanced during freezing (Lawrie, 1968).

Meat protein denaturation takes place during a freezing process, it is accompanied by conformational changes in a structure of a protein molecule. Since denaturation ruptures large number of weak hydrogen and then hydrophobic and ionic bonds, it should be assumed that cryodenaturation destroys mainly secondary structure of proteins. This structure is formed by an interaction between functional groups of amino acids by hydrogen bonds between the oxygen atoms and amino groups nitrogen atoms.

Table 2. Changes in the free amino acids contents in veal after freezing depending on the polarity of radicals

Amino acid	Functional group	Mass fraction of free amino acids in meat, mg g(protein)$^{-1}$		
		before freezing	after freezing at t, °C	
			-24	-35
Nonpolar amino acids with hydrophobic side chains				
Alanine	Aliphatic hydrocarbon R-groups	1.11 ± 0.08	1.7 ± 0.1	2.4 ± 0.2
Glycine		0.61 ± 0.06	0.74 ± 0.05	0.76 ± 0.06
Valine		0.24 ± 0.02	0.49 ± 0.03	0.4 ± 0.03
Leucine		0.31 ± 0.02	0.64 ± 0.04	0.44 ± 0.04
Isoleucine		0.17 ± 0.01	0.3 ± 0.03	0.25 ± 0.02
Proline		0.17 ± 0.01	0.24 ± 0.02	0.19 ± 0.01
Hydroxyproline		0.037 ± 0.003	0.042 ± 0.003	0.032 ± 0.002
Total:		2.65	4.15	4.47
Phenylalanine	Aromatic, heterocyclic hydrocarbon R-groups	0.2 ± 0.02	0.31 ± 0.02	0.24 ± 0.02
Tryptophan		trace amounts	trace amounts	trace amounts
Total:		0.20	0.31	0.24
Methionine	-SH	0.021 ± 0.001	0.042 ± 0.004	0.038 ± 0.002
Total:		0.021	0.042	0.038
In total:		2.87	4.50	4.75
Polar amino acids with hydrophilic non-ionogenic radicals				
Tyrosine	-OH	0.19 ± 0.01	0.39 ± 0.03	0.29 ± 0.02
Serine		0.34 ± 0.02	0.60 ± 0.04	0.41 ± 0.04
Threonine		0.29 ± 0.02	0.77 ± 0.05	0.59 ± 0.05
Cysteine + Cystine	-SH	trace amounts	trace amounts	trace amounts
Total:		0.82	1.76	1.29
Polar amino acids with anionic radicals				
Aspartic acid & Asparagine	-COOH	2.6 ± 0.2	1.9 ± 0.2	1.54 ± 0.08
Glutamic acid & Glutamine		2.4 ± 0.1	1.41 ± 0.08	1.2 ± 0.1
Total:		5.0	3.31	2.74
Polar amino acids with cationic radicals				
Histidine		1.47 ± 0.06	2.2 ± 0.2	1.15 ± 0.08
Lysine		0.18 ± 0.01	0.76 ± 0.08	0.63 ± 0.05
Arginine		11.0 ± 0.8	15.2 ± 0.9	11.7 ± 0.6
Total:		12.65	18.16	13.48

Hydrogen bonds are formed between uncharged hydrophilic groups (-OH, -CO-NH2, SH-groups) and any other hydrophilic groups. Secondary structure of proteins has regular structures of two types: α-helix or β-structure. α-helical structure is formed by a great amount of hydrogen bonds and it is among the most stable conformation of the peptide backbone corresponding to the free energy minimum.

As a result of α-helix formation the polypeptide chain is shortened, but during cryodenaturation due to rupture of hydrogen bonds, the polypeptide chain elongates and becomes more accessible to the action of enzymes, as evidenced by the increase in free amino acids content (Table 1, 2).

In contrast to α-helices, breaking of hydrogen bonds that form β-structure by lots of hydrogen bonds between peptide groups linear regions of a single polypeptide chain or between different polypeptide chains does not cause elongation of the latter. Consequently the action of enzymes in β-structure of the denatured protein molecules will be hindered.

It should be noted that a possible cryodenaturation result is violation of the tertiary structure of proteins formed by hydrophobic, ionic and hydrogen bonds, but not covalent ones. Hydrophobic interactions occur between hydrophobic amino acid radicals, as well as Van der Waals forces between the closely spaced to each other atoms. As a result, hydrophobic core is formed inside of the protein globule. In the denatured protein hydrophobic radicals that in the native molecule structure are hidden within a hydrophobic core appear on the surface. In absence of strong repulsive charge molecules associate with each other by hydrophobic bonds that result in decreased proteins solubility. In addition, the compact dense spatial structure of the native protein after cryodenaturation is considerably increased in size and becomes also easily accessible to the action of enzymes.

Hydrophilic groups of amino acid radicals are also involved in the formation of the protein molecules tertiary structure. They tend to form hydrogen bonds with water, and therefore, they are mainly located on the surface of the protein molecule.

All hydrophilic amino acid group radicals trapped within the hydrophobic core interact with each other through ionic and hydrogen bonding. Ionic bonds occur generally between charged (anionic) carboxyl groups of aspartic and glutamic acids and the positively charged (cationic) groups of lysine, arginine or histidine.

Changes in proteins amino acids after freezing

The total amount of essential and nonessential amino acids and amount of each amino acid of veal protein are reduced during the freezing process regardless of temperature. However, such changes depend considerably on the structural characteristics of amino acids, chemical structure of their radicals and their solubility in water as well as on the freezing temperature.

Thus, the amount of essential amino acids of veal frozen at temperatures of -24°C and -35°C decreased by 10% and 7%, non-essential – by 17% and 9%, respectively (Table 3).

Table 3. Changes of essential and non-essential amino acids contents in veal proteins after freezing

Amino acid	Mass fraction of amino acids in meat, mg g(protein)$^{-1}$		
	before freezing	after freezing at t, °C	
		-24	-35
Essential			
Histidine	17.7 ± 1.8	13.2 ± 0.9	14.6 ± 1.3
Isoleucine	49 ± 5	40 ± 4	43.4 ± 3.1
Leucine	48 ± 4	46.6 ± 2.2	47 ± 4
Lysine	102 ± 7	91 ± 10	90 ± 5
Methionine	40 ± 4	36.4 ± 3.0	36.7 ± 3.2
Cysteine + Cystine	21.0 ± 2.0	20.4 ± 0.9	20.8 ± 1.9
Phenylalanine	44.3 ± 3.9	40 ± 4	43.1 ± 3.5
Tyrosine	32.8 ± 1.4	30.6 ± 3.1	31.4 ± 1.7
Threonine	33.3 ± 1.9	24.6 ± 2.2	28.7 ± 2.8
Tryptophan	22.4 ± 2.4	19.7 ± 1.2	21.0 ± 1.7
Valine	67 ± 4	65 ± 5	66 ± 6
Total:	477.5	427.5	442.7
Non-essential			
Alanine	32.3 ± 2.6	28.7 ± 1.6	30.4 ± 2.8
Arginine	52 ± 5	38.5 ± 1.6	43.4 ± 2.7
Serine	16.2 ± 1.0	13.5 ± 1.1	14.2 ± 1.4
Aspartic acid & Asparagine	47.4 ± 3.8	36.5 ± 1.8	39 ± 4
Glutamic acid & Glutamine	39.1 ± 3.5	28.7 ± 2.8	32.6 ± 1.5
Glycine	72 ± 7	68.3 ± 3.7	70.4 ± 3.8
Proline	26.1 ± 1.8	22.5 ± 1.4	24.6 ± 1.6
Hydroxyproline	62 ± 4	50.6 ± 3.7	60 ± 5
Total:	347.1	287.3	314.6
In total:	824.6	714.8	757.3

Table 4 shows that the freezing process and its temperature have least effect on changes of amino acids with a nonpolar (hydrophobic) side chain group type compared to other studied amino acids. Their content decreases in veal frozen at -24°C and -35°C by 10% and 4%, respectively.

It is known that non-polar radicals having aliphatic hydrocarbon chains (radicals of alanine, valine, leucine, isoleucine, proline and methionine) and aromatic rings (radicals of phenylalanine and tryptophan) tend to each other or to other hydrophobic molecules in water, reducing their contact surface with water (Baynes & Dominiczak, 2009). So it can be assumed that freezing of moisture has no significant effect on deep proteolysis of proteins associated with the cleavage of amino acids with hydrophobic radicals.

Table 4. Changes in the amino acids contents in veal after freezing depending on the polarity of radicals

Amino acid	Functional group	Mass fraction of amino acids in meat, mg g(protein)$^{-1}$		
		before freezing	after freezing at t, °C	
			-24	-35
Nonpolar amino acids with hydrophobic side chains				
Alanine	Aliphatic hydrocarbon R-groups	32.3 ± 2.6	28.7 ± 1.6	30.4 ± 2.8
Glycine		72 ± 7	68.3 ± 3.7	70.4 ± 3.8
Valine		67 ± 4	65 ± 5	66 ± 6
Leucine		48 ± 4	46.6 ± 2.2	47 ± 4
Isoleucine		49 ± 5	40 ± 4	43.4 ± 3.1
Proline		26.1 ± 1.8	22.5 ± 1.4	24.6 ± 1.6
Hydroxyproline		62 ± 4	50.6 ± 3.7	60 ± 5
Total:		356.4	321.7	341.8
Phenylalanine	Aromatic, heterocyclic hydrocarbon R-groups	44.3 ± 3.9	40 ± 4	43.1 ± 3.5
Tryptophan		22.4 ± 2.4	19.7 ± 1.2	21.0 ± 1.7
Total:		66.7	59.7	64.1
Methionine	-SH	40 ± 4	36.4 ± 3.0	36.7 ± 3.2
Total:		40	36.4	36.7
In total:		463.1	417.8	442.6
Polar amino acids with hydrophilic non-ionogenic radicals				
Tyrosine	-OH	32.8 ± 1.4	30.6 ± 3.1	31.4 ± 1.7
Serine		16.2 ± 1.0	13.5 ± 1.1	14.2 ± 1.4
Threonine		33.3 ± 1.9	24.6 ± 2.2	28.7 ± 2.8
Cysteine + Cystine	-SH	21.0 ± 2.0	20.4 ± 0.9	20.8 ± 1.9
Total:		103.3	89.1	95.1
Polar amino acids with anionic radicals				
Aspartic acid & Asparagine	-COOH	47.4 ± 3.8	36.5 ± 1.8	39 ± 4
Glutamic acid & Glutamine		39.1 ± 3.5	28.7 ± 2.8	32.6 ± 1.5
Total:		86.5	65.2	71.6
Polar amino acids with cationic radicals				
Histidine		17.7 ± 1.8	13.2 ± 0.9	14.6 ± 1.3
Lysine		102 ± 7	91 ± 10	90 ± 5
Arginine		52 ± 5	38.5 ± 1.6	43.4 ± 2.7
Total:		171.7	142.7	148.0

Amino acids with uncharged hydrophilic radicals undergo more changes than ones with nonpolar radicals. Radicals of these acids better dissolve in water, since they are composed of polar functional groups (hydroxyl in serine, threonine and tyrosine, thiol in cysteine) that form hydrogen bonds with water. In this regard, water freezing and proteins denaturation followed by a break first of all of weak hydrogen bonds lead to a decrease of these acids amounts during freezing.

Amount of amino acids with polar non-ionogenic radicals is reduced by 14% and 8% in veal proteins during freezing at temperatures of -24°C and -35°C, respectively.

Significant content changes were noted for amino acids with polar anionic and cationic radicals. Amounts of amino acids with negatively charged polar radicals – aspartic acid and asparagine, glutamic acid and glutamine, after veal freezing at -24°C are reduced by 23% and 27%, after freezing at -35°C – by 18% and 17%, respectively.

The total content of amino acids with polar positively charged cations (histidine, lysine, arginine) in veal frozen at temperatures of -24°C and -35°C decreased by 17% and 14%, respectively.

The contents of amino acids such as tyrosine, leucine, valine and cysteine do not change at studied veal freezing temperatures, obviously due to their low solubility in water. In this regard, water freezing does not affect their structural changes.

Amino acid score of indispensable amino acids was calculated to estimate the changes in the biological value of veal after freezing (Table 5). It was achieved by a comparison of the content of the amino acid in the protein with its content in the requirement pattern (Eq 1) (FAO/WHO, 2007).

$$\text{Amino acid score} = \frac{\text{mg of amino acid in 1 g test protein}}{\text{mg of amino acid in requirement pattern}} \qquad (1)$$

Table 5. Changes in amino acid score of veal proteins after freezing

Amino acid	Adult requirements, mg g(protein)$^{-1}$ (FAO/WHO, 2007)	Amino acid score		
		before freezing	after freezing at t, °C	
			-24	-35
Histidine	15	1.18	0.88	0.97
Isoleucine	30	1.63	1.33	1.45
Leucine	59	0.81	0.79	0.80
Lysine	45	2.27	2.02	2.00
Methionine	16	2.50	2.28	2.29
Cysteine	6	3.50	3.40	3.47
Phenylalanine + tyrosine	38	2.03	1.86	1.96
Threonine	23	1.45	1.07	1.25
Tryptophan	6	3.73	3.28	3.50
Valine	39	1.72	1.67	1.69

CONCLUSIONS

The content of most free amino acids has increased in veal after freezing and defrosting. The greatest changes took part in the free polar amino acids with hydrophilic nonionogenic and cationic radicals. The amount of free amino acids with hydroxyl nonionogenic radicals increased 2.15 and 1.57 times after freezing at -24°C and -35°C, respectively. The content of free amino acids with cationic radicals increased 1.44 times in veal, frozen at -24°C. Accumulation of free amino acids during meat freezing is associated with the proteolysis of muscle and connective tissue proteins.

Freezing temperature has a weak effect on the total content of amino acids with hydrophobic (nonpolar) radicals in veal. Their content decreases by 10% and 4% after freezing at -24°C and -35°C, respectively. Significant changes are established for amino acids with polar anionic and cationic radicals during veal freezing at -24°C. The content of amino acids with polar anionic radicals decreased by 25%, with cationic radicals – by 17%. This can probably be explained by the reaction of decarboxylation and the formation of nitrogenous compounds.

It was found that the veal freezing process at temperatures -24°C and -35°C reduces amino acid score of all essential amino acids, although to varying degrees. Median amino acid score decrease of 10% occurred after freezing at -24°C (minimum decrease of 2% and maximum of 26%). After freezing at -35°C median amino acid score decrease was 7% (minimum–1%, maximum–18%). In cooled veal the only limiting amino acid was leucine with amino acid score of 0.81. There were two limiting amino acids in veal after freezing – leucine and histidine. Their scores after freezing at -24°C were 0.79 and 0.88, at -35°C – 0.80 and 0.97, respectively.

It is recommended to freeze veal rapidly at -35°C and below to slow the proteolysis and save contents of amino acids with polar nonionic, anionic and cationic radicals.

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REFERENCES

Baynes, J. & Dominiczak, M. H. 2009. *Medical biochemistry*. Elsevier Health Sciences, 712 pp.

Benjakul, S., Visessanguan, W., Thongkaew, C. & Tanaka, M. 2003. Comparative study on physicochemical changes of muscle proteins from some tropical fish during frozen storage. *Food Res. Int.* **36**(8), 787–795.

Bernaś, E. & Jaworska, G. 2012. Effect of preservation method on amino acid content in selected species of edible mushroom. *LWT-Food Sc. and Tech.* **48**(2), 242–247.

Bhatnagar, B.S., Bogner, R.H. & Pikal, M.J. 2007. Protein stability during freezing: separation of stresses and mechanisms of protein stabilization. *Pharm. Dev and Tech.* **12**(5), 505–523.

Chang, B.S., Kendrick, B.S. & Carpenter, J.F. 1996. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. *J. of Pharm. Sc.* **85**(12), 1325–1330.

FAO. 1970. *Amino-Acid content of foods and biological data on proteins.* FAO food and nutrition series. Rome, Italy: FAO.

FAO/WHO. 1991. Protein Quality Evaluation: Report of the Joint FAO/WHO Expert Consultation. *FAO food and nutrition paper* **51**.

FAO/WHO. 2007. *Protein and amino acid requirements in human nutrition. Report of a joint WHO/FAO/UNU expert consultation*. Geneva, Switzerland: World Health Organization, WHO technical report series no. 935.

FAO/WHO. 2011. Dietary protein quality evaluation in human nutrition. *FAO food and nutrition paper* **92**.

Fierabracci, V., Masiello, P., Novelli, M. & Bergamini, E. 1991. Application of amino acid analysis by high-performance liquid chromatography with phenyl isothiocyanate derivatization to the rapid determination of free amino acids in biological samples. *J. of Chrom. B: Biomed. Sc. and App.* **570**(2), 285–291.

González-Castro, M.J., López-Hernández, J., Simal-Lozano, J. & Oruna-Concha, M.J. 1997. Determination of amino acids in green beans by derivatization with phenylisothiocianate and high-performance liquid chromatography with ultraviolet detection. *J. of Chrom. Sc.* **35**(4), 181–185.

Gunawan, S., Walton, N.Y. & Treiman, D.M. 1990. High-performance liquid chromatographic determination of selected amino acids in rat brain by precolumn derivatization with phenylisothiocyanate. *J. of Chrom. A*, **503**, 177–187.

Heinrikson, R.L. & Meredith, S.C. 1984. Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. *An. Biochem.* **136**(1), 65–74.

Khan, A.W. & Berg, L. 1967. Biochemical and quality changes occurring during freezing of poultry meat. *Journ. of Food Sc.* **32**(2), 148–150.

Khan, A.W., Davidkova, E. & Berg, L. 1968. On cryodenaturation of chicken myofibrillar proteins. *Cryobiol.* **4**(4), 184–188.

Lawrie, R.A. 1968. Chemical changes in meat due to processing — A review. *J. of the Sc. of Food and Agr.* **19**(5), 233–240.

Mitić, S. 1976. Transformation of amino acid composition in bacterial cells of *Lactobacillus bulgaricus* during freeze-drying. *Cryobiol.* **13**(2), 214–217.

Riss, T.L., Bechtel, P.J., Forbes, R.M., Klein, B.P. & McKelth, F.K. 1983. Nutrient content of special fed veal ribeyes. *J. of Food Sc.* **48**(6), 1868–1869.

Wagner, J.R. & Anon, M.C. 1985. Effect of freezing rate on the denaturation of myofibrillar proteins. *Int. J. of Food Sc. & Tech*. **20**(6), 735–744.

Williams, A.P. 1978. The amino acid, collagen and mineral composition of preruminant calves. *The J. of Agr. Sc.* **90**(03), 617–624.

Williams, P. 2007. Nutritional composition of red meat. *Nutr. & Diet.* **64**(s4), S113–S119.

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Vegetable ingredients in soft cheese made from concentrated skim milk by ultrafiltration

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Abstract. The objective of this research was to develop the technology and composition of soft cheese made from concentrated skimmed milk by ultrafiltration with a low concentration factor. UF enables reduction in the quantities of starter, rennet, colorants, and cheese making costs per vat. The use of UF milk appeal to lactose intolerant consumers because of the low lactose levels in the product.

Ultrafiltration was carried out using polyethersulfone membranes. Jerusalem potato and carrot were chosen as vegetable ingredients. Since the soft cheese was made from skimmed milk retentate, there was a necessity to compensate the lack of fat. In this research the impact of orange fiber on organoleptic qualities was determined. For imparting mouthcoating, creaminess, and providing for an even meltaway effect in the mouth CITRI–FI 100 FG (pure orange fiber), CITRI–FI 200 FG (orange fiber with guar gum), CITRI–FI 300 FG (orange fiber with xanthan gum) were chosen. Application of CITRI–FI 200 FG and CITRI–FI 300 FG enables the production of high quality curd only up to certain concentrations, which do not have a significant impact on the organoleptic qualities of soft cheese. The use of CITRI–FI 100 FG enables to get firm curd and can at the same time create organoleptic properties of a fat-containing product in the finished soft cheese.

Thus, on the basis of data CITRI–FI 100 FG is recommended to use for manufacturing soft cheese where mouthfeel is needed. Optimal concentrations of CITRI–FI 100 FG were determined.

Key words: Ultrafiltration, Jerusalem potato, soft cheese, vegetable ingredients, orange fiber.

INTRODUCTION

Due to the present unfavourable environmental and economic situation, the dietary patterns of the population are undergoing a significant change towards an imbalance of major components in diet (Bogatyrev, 1985; Bobylin et al., 2000). Thus, the main problem in the field of nutrition is to create a range of products promoting improvement of human health in daily consumption. Our research tries to solve this problem by creating products with both dairy and non-dairy components. These components help to improve food and the biological values of the products.

Nowadays, with increasing concern about consumer health, the aim is to reduce fat and calorie consumption. Low fat, low calorie foods, which look and taste like their fatty counterparts higher in calories, have been gaining in popularity. Thus, it is topical to concentrate on the development of food products, which are nutritious and contain reduced levels of calories and fat.

By analyzing international experience, it was determined that increasing production and expanding the range of soft cheeses could be a good idea. Along with the high biological value, this type of cheese can be sold without ripening. Despite the perspective in the manufacture of soft cheese, most of the whey proteins are lost in the whey.

Ultrafiltration has been used in cheese making since about 1971 (Maubois & Mocquot, 1971). Eventually the use of ultrafiltration has gained significance in cheese making industry. Ultrafiltration enables the concentration, separation, and recovery of individual milk components (Salhab, 1998). It provides complete retention of whey proteins and improvement of the traditional technological process of manufacturing cheese (Lelievre & Lawrence, 1988).

Low concentration factor (CF) of skimmed milk permits the use of traditional equipment for cheese making and the only investment expenses are the purchase of an ultrafiltration plant and putting it in service. Ultrafiltration can help minimize seasonal variations in milk composition, which, in turn, can help standardize rennet coagulation time, gel strength, and cheese yield (Lucey, 2000).

Based on the analysis of information data, Jerusalem potato and carrot purée have been chosen as the vegetable ingredient. Jerusalem potato is a source of inulin and oligofructose. Inulin and oligofructose improve immunity as well as calcium absorption, reduce cholesterol in the blood and even reduce the risk of colon cancer (Davidovich, 1957; Perkovets, 2007). Jerusalem artichoke has an ability of not accumulating heavy metals (Reshetnik et al., 1997).

Carrot is used in cases of anemia, scurvy, rheumatism, visual disturbances, cardiovascular diseases, and loss of strength. Carrot has hematopoietic, bactericidal, anti-inflammatory, choleretic, diuretic, laxative, analgesic, and anti-sclerotic effect (Novichikhina, 2003).

Orange fiber is a natural fiber, derived from the cell tissues of dried orange pulp without using chemical reagents and only by mechanical treatment. It has a positive effect on the physiological processes of the human organism: purifies from slags, reduces cholesterol, eliminates heavy metals and improves the functioning of the gastrointestinal tract (Gubina, 2010).

Based on the foregoing, the objective of this research was to develop the composition of soft cheese made from concentrated skimmed milk by ultrafiltration, using vegetable components.

MATERIAL AND METHODS

Preparation of retentate

Whole cow milk was obtained from a local market. Fat was removed by mechanical separation that resulted in skimmed milk (8.65% non-fat solids, 3.19% total protein, 0.09% fat) and cream. Before concentration the skimmed milk was heated to 65°C and held at that temperature during 25 s. The skimmed milk was concentrated by using laboratory scale set Vivaflow 50. (‘Vivascience’, Sartorius group) (Fig. 1). Ultrafiltration was carried out using polyethersulfone membranes (molecular weight cut-off of 30 kDa, pump flow 200–400 ml min^{-1}), until a retentate with CF 2 was obtained. The temperature during the process was 50°C. Polyethersulfone membranes are preferred

for their low fouling characteristics, broad pH range, and durability. They also exhibit no hydrophobic or hydrophilic interactions.

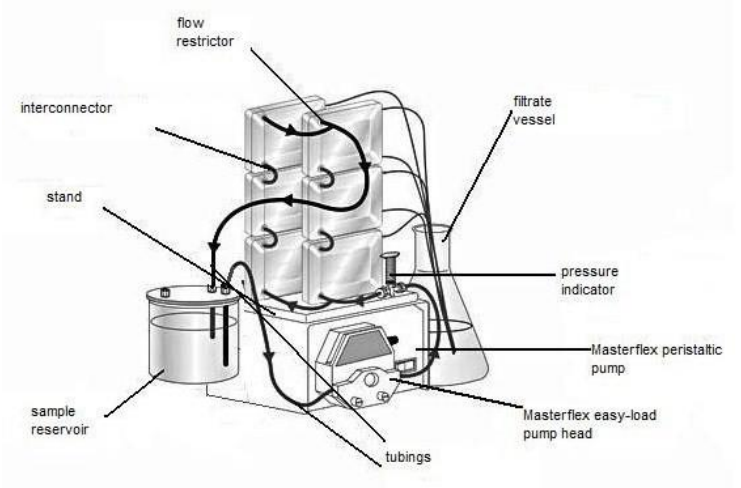

Figure 1. Vivaflow 50 set up.

Starter culture: BC–Uglich–No 4 (*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Leuconostoc lactis*).

Ferment: Calf rennet was obtained from Moscow rennet factory.

Jerusalem potato: Powder of Jerusalem potato was obtained from Seliger.

Carrot purée: Sterilized carrot purée was obtained from a local store.

CITRI-FI: Orange fiber was obtained from Fiberstar Inc., USA.

Non–fat solids, total protein, and fat of skimmed milk were measured using milk analyzer 'Klever–2'.

To determine the concentrations of Jerusalem potato suitable for cheese manufacturing, retentate was divided into several parts. Each part (1 l.) with added Jerusalem potato was heated at 74 ± 2°C for 20–25 s. and cooled to 29°C. The doses of Jerusalem potato ranged from 1% to 5% of retentate weight with the increment of 1%. The control sample contained no vegetable component. Starter culture prepared by inoculating sterilized skimmed milk with culture was added in the amount of 1%. *Lactococcus lactis* subsp. *lactis* is used as an active acidifier. *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Leuconostoc lactis* are very important for the flavour of the cheese. During the fermentation of starter culture, lactic acid accumulates, which causes the pH–value to decrease, resulting in better whey drainage. The mixtures were coagulated with calf rennet and left to curdle. After coagulation the curds were subjected to organoleptic assessment. Jerusalem potato was chosen because of its medicinal properties, specific pleasant taste qualities, and medical recommendation for consumption. But adding this component resulted in cheese with unacceptable colour for consumers. Carrot purée was decided to add for correction.

Investigations for determining the concentrations of sterilized carrot purée were carried out according to the above procedure with only one addition, so that carrot purée was added after the pasteurization of the mixture. The concentrations of Jerusalem potato were 2% and 3%. The concentrations of sterilized carrot purée ranged from 1% to 9% with the increment 1%.

Despite the fact that inulin and oligofructose in Jerusalem potato have the capacity of being used as fat replacers with excellent mouthfeel characteristics and provide better organoleptic properties, their concentrations in the product are insufficient to get soft cheese with the appearance, taste, consistency, and texture of fat–containing cheese.

To maintain a desirable creamy mouthfeel characteristic of the product, orange fiber was chosen. This fiber is a powder of light cream colour with a neutral taste and smell. As fiber does not require pre-hydration, it was added in the mixture in different concentrations together with the other dry ingredient before heat treatment. The following additives recommended by the manufacturer were chosen: CITRI–FI 100 FG (pure orange fiber), CITRI–FI200 FG (orange fiber with guar gum), and CITRI–FI 300 FG (orange fiber with xanthan gum). To determine the possible use of the additive, samples of soft cheeses were obtained with additive dose ranging from 0.05% to 0.4% of mixture weight with the increment of 0.05%. After complete coagulation, the curds were cut into cubes, which were settled for 10 min. The curds were stirred carefully during the next 20 min and settled for 15 min. After that, the whey was drained and curds were transferred into perforated moulds for draining and pressed under the force of gravity. The samples of cheese were turned upside down three times during the first 5 h of draining and pressing. The pressing temperature was 16–18°C. Then the cheeses were soaked in concentrated brine solution (18–22%) for salting for an appropriate period of time depending on the size of the cheese sample.

Organoleptic assessment

Samples were evaluated for organoleptic properties by a taste panel of the 11 staff members and students from Milk Technology and Food Biotechnology Department. The participants were selected and trained in accordance with the ISO 8586-1 standard (1993). Requirements for the work of the group of assessors were according to ISO 8589 standard (2007).

Organoleptic evaluation of cheese curds

The aim of the cheese curds sensory evaluation was to determine the acceptable concentrations of vegetable additive for the manufacturing of soft cheese. The quality of the cheese curds was evaluated for appearance (colour, colour homogeneity), consistency, and texture (hardness, and flavour (odour and taste), using a 7–point scale (Pereira et al., 2011). The participants were asked to assess a number of specific attributes (Tables 1 and 2). The cheese curds were randomly coded with three-digit numbers.

Table 1. Description of organoleptic attributes for several sensory features, used by selected assessors to assess cheese curds with various concentrations of Jerusalem potato

Sensory features	Organoleptic attribute	Continuous scale	
		0	7
Appearance	Colour	White	Dark beige
Taste and odour	Intensity of Jerusalem potato taste and odour	Not detected	Pronounced
	Creaminess	Not detectable	Intensive
	Aftertaste	Not intensive	Intensive
Consistency and texture	Firmness	Weak, very soft	Very firm

Table 2. Description of organoleptic attributes for several sensory features, used to assess cheese curds with concentrations of Jerusalem potato (2% and 3%) and various concentrations of sterilized carrot purée

Sensory features	Organoleptic attribute	Continuous scale	
		0	7
Appearance	Colour	Gray beige	Creamy
	Colour homogeneity	Heterogeneous	Homogeneous
Taste and odour	Intensity of Jerusalem potato taste and odour	Slight	Pronounced
	Creaminess	Detectable	Intensive
	Aftertaste	Slight	Very intensive
Consistency and texture	Firmness	Weak, very soft	Very firm

Cheese organoleptic evaluation

The aim was to compare more than two samples; therefore, the method of ordering by preference was applied. The samples were coded with three-digit random numbers. A cheese sample was divided into various portions and equilibrated at room temperature. Its overall acceptability was evaluated, using a five–point hedonic scale (from 1 = I disliked very much to 5 = I liked very much). The samples were presented to the group in randomized order after salting. For assessment were chosen samples with CITRI–FI concentrations that allowed to obtain firm cheese curds with a sharp, clean split which were suitable for manufacturing cheeses with more or less improved organoleptic quality.

pH measurement

pH values were measured using pH–meter (pH 4–10 with a glass combination electrode).

Moisture measurement

Moisture content was determined by drying method using moisture determining device 'ELEX – 7'.

RESULTS AND DISCUSSIONS

The results of the sensory assessment of cheese curds quality are given in Figs. 2–4.

It can be seen (Fig. 2) that appearance, taste and odour, consistency, and texture of cheese curds were affected by the concentrations of Jerusalem potato.

The control cheese curd was a little firmer than the cheese curds with increasing concentration of Jerusalem potato. The colour range of the control sample and the samples with Jerusalem potato addition varied significantly. The concentration increase up to 3% led to insignificant decrease in curd firmness. The increase in concentrations of vegetable additive more than 3% led to a gradual decrease in organoleptic characteristics. The consistency of cheese curds became weaker, porous and even deliquescent. The taste and flavour of Jerusalem potato were strongly pronounced. All that makes these cheese curds the least acceptable for manufacturing cheese.

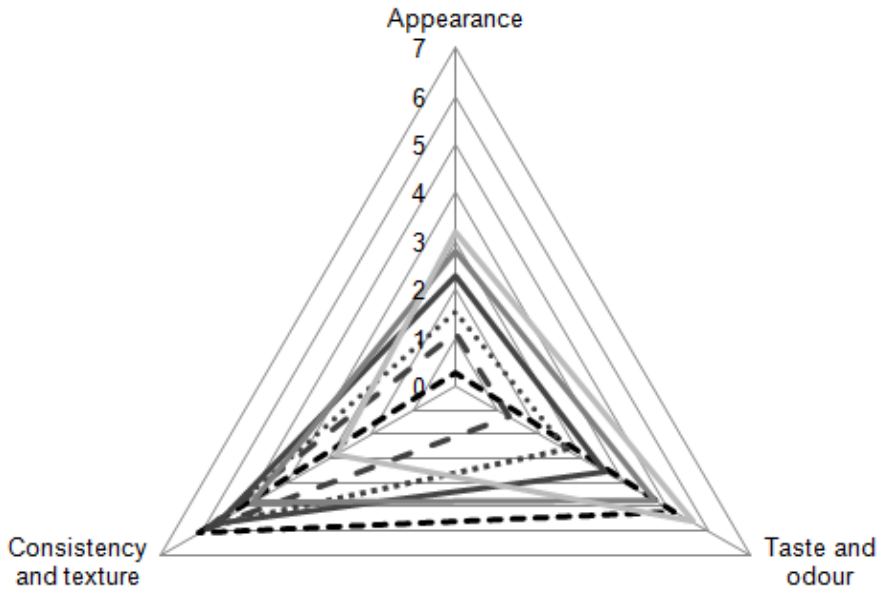

Figure 2. Spider plot of sensory attributes (lowest = 0; highest = 7) of cheese curds samples with various concentrations of Jerusalem potato, where: ---- control sample, – – 1%, ····· 2%, —— 3%, —— 4%, —— 5%.

Thus, the most acceptable concentrations of Jerusalem potato for cheese manufacturing are in the range from 2% to 3%. Further concentration increase leads to deterioration in the consistency of the curds, which complicates obtaining soft cheeses and makes them unacceptable for the consumer.

The data obtained from the profiles (Figs. 3, 4) showed that with the increase in carrot purée concentration and constant Jerusalem potato concentration, the cheese curd appearance became better. The samples gradually obtained a creamy colour. At the same time, an increase in carrot purée concentration resulted in a decrease in the consistency quality of the curd. When comparing the samples, it was found out that the higher the concentration of carrot purée, the lower the intensity of Jerusalem potato taste and odour, creaminess, and aftertaste in the cheese curd samples.

Based on the data, the recommended concentration of Jerusalem potato is no more than 2%, while for sterilized carrot purée the concentration should be no more than 8%. The optimal concentration of carrot purée is 8%, as this concentration improves colour and positively influences the taste of the product. With the increase in concentration of Jerusalem potato more than 2% and sterilized carrot purée more than 8%, deterioration in the quality of the curd occurred.

Research on laboratory scale has established relationships between concentrations of orange fiber and organoleptic characteristics of the soft cheese. The increase in the amount of CITRI–FI 300 FG has a negative effect on the quality of the curds. Acceptable curds may be obtained if the amount of the additive does not exceed 0.05 % of the weight of the mixture. But this amount has no positive effect on the organoleptic characteristics of the final product. With increasing dosage of CITRI–FI 300 FG, curds became mushy

and with a ragged split, making the further process of obtaining a product with acceptable organoleptic characteristics complicated.

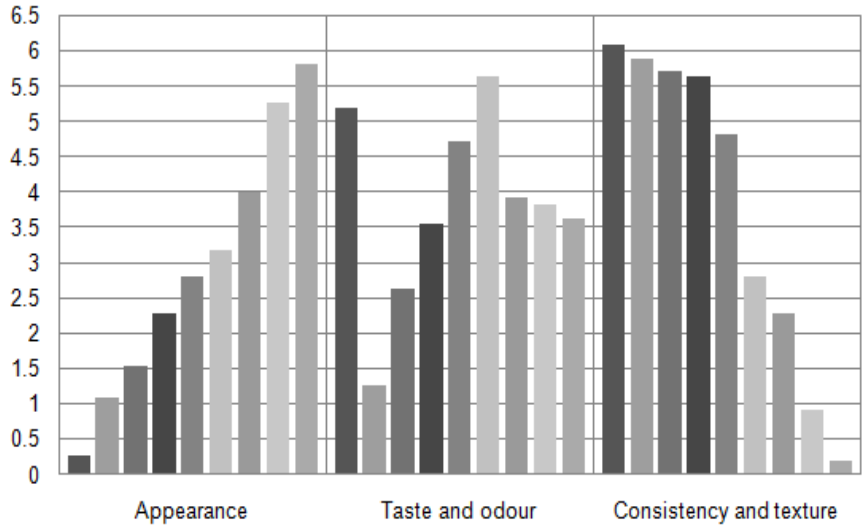

Figure 3. Descriptive sensitive profiles for samples with 3% of Jerusalem potato and various concentrations of carrot purée, where: ■ 1%, ■ 2%, ■ 3%, ■ 4%, ■ 5%, ■ 6%, ■ 7%, ■ 8%, ■ 9%.

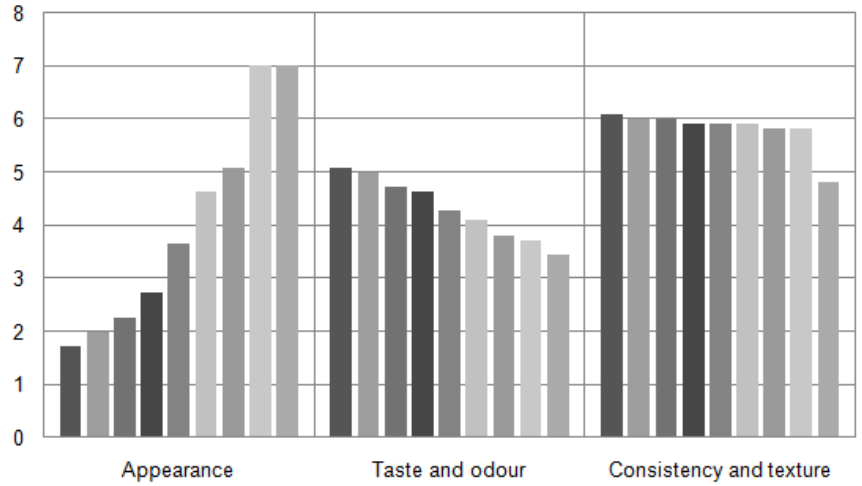

Figure 4. Descriptive sensitive profiles for samples with 2% of Jerusalem potato and various concentrations of carrot purée, where: ■ 1%, ■ 2%, ■ 3%, ■ 4%, ■ 5%, ■ 6%, ■ 7%, ■ 8%, ■ 9%.

The application of CITRI–FI 200 FG to improve the organoleptic characteristics of cheese revealed that an increase in the amount of additive has a negative impact on the taste and consistency of the obtained curds, and hence on the organoleptic characteristics of the product. The concentration of the additive more than 0.25 % of the weight of the mixture resulted in porous consistency of the curds, which further resulted in large losses of solids in the whey. With increasing dosage of CITRI–FI 200 FG, the finished product obtains an off-flavour. The amount of the additive lower than 0.25 % did not lead to significant improvement in the taste characteristics of the final product.

The application of CITRI–FI 100 FG at an approximate level of 0.05% to 0.4% enables to obtain curds with a sharp, clean split. The finished product has gained creamy mouth feel characteristics and an acceptable texture. Based on the sensory characteristics of the soft cheese, the addition of CITRI–FI 100 FG to the cheeses at levels 0.25 % or 0.3% of mixture weight was preferred. Further increase does not lead to significant improvement in the quality of the finished product.

For providing a soft cheese with the texture, smoothness, and organoleptic properties of a fatty product, the possibility of using CITRI–FI was investigated. The positive data were obtained. The researches on the effect of CITRI–FI concentrations on pH value of the soft cheeses were carried out. The data are presented in Fig. 5.

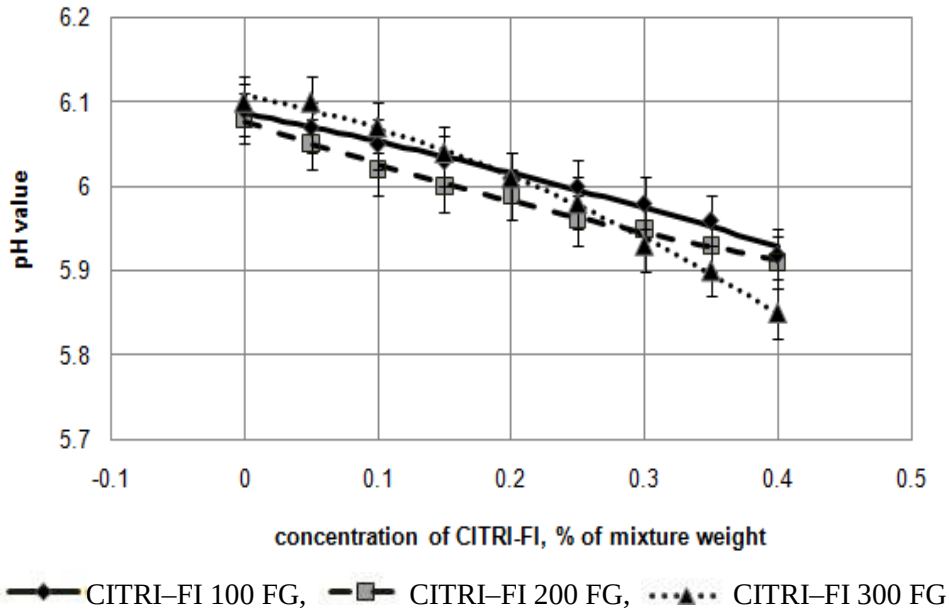

Figure 5. Effect of CITRI–FI concentrations on pH value of the soft cheeses.

The overall quality of cheese samples (Fig. 6) with the addition of CITRI–FI 100 (0.25%) was more acceptable for the panelists than control samples without CITRI–FI and with concentrations of CITRI–FI 300F G (0.05%) and CITRI–FI 200 FG.

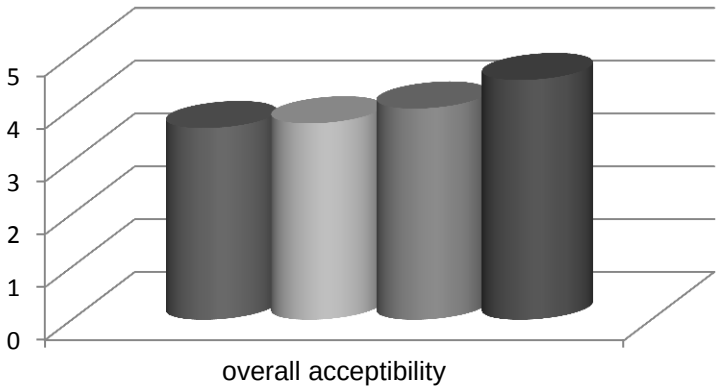

Figure 6. Overall quality of the products investigated, where: ■ control sample, ■ CITRI–FI 300FG, ■ CITRI–FI 200FG, ■ CITRI–FI 100FG.

CONCLUSIONS

1. In the course of the production of soft cheese from ultrafiltrated skimmed milk, Jerusalem potato and carrot purée can be used as vegetable ingredients with dosage 2% and 8%, respectively.

2. In order to produce soft cheese with the texture, smoothness and organoleptic properties of a fatty product, but containing no substantial fat, it is recommended to use CITRI–FI 100 FG at a level 0.25%.

3. The increase in CITRI-FI concentrations was stated to reduce the pH value of the soft cheese inconsiderably.

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REFERENCES

Bobylin, V.V., Ostroumova, T.A. & Braginsky, V.I. 2000. Scientific fundamentals of manufacture of combined soft cheeses. *International scientific-practical conference 'Environmental, technological and economic aspects of food production*, Semey, pp. 99–100 (in Russian).

Bogatyrev, G.N. 1985. Designing of Food. *Chemistry and Life.* **12**, 3–9 (in Russian).

Davidovich, S.S. 1957. *Jerusalem potato.* State Publishing House of Agricultural Literature, Moskva, 92 pp. (in Russian).

Gubina, I. 2010. 'CITRI-FI' – a new component of a healthy diet. *Milk Processing* **3,** 51.

ISO 8586 – 1:1993. Sensory analysis. General guidance dor the selection, training and monitoring of assessors, Part 1. Selected assessors.

ISO 8589:2007. Sensory analysis–General guidance for the design of test rooms.

Lelievre, J. & Lawrence, R.C. 1988. Manufacture of cheese from milk concentrated by Ultrafiltration. *J. Dairy Res*. **55**, 465–478.

Lucey, J. 2000. Dairy ingredients in cheese making–possibilities and problems. *Dairy pipeline*, **12**(2), 1, 4.

Maubois, J.L. & Mocquot, G. 1971. The assesment of cheesemaking profitability. *Milk*. **51**, 416–420.

Novichikhina, L.I. 2003. *Green Pharmacy on doc. Mittlayder's garden beds.* Minsk, 351 pp. (in Russian).

Perkovets, M.V. 2007. Dairy products with inulin and oligofructose. *Dairy Industr*. **11**, 64–66 (in Russian).

Pereira, C.I., Franco, M.I., Gomes, A.M.P. & Malcata, F.X. 2011. Microbiological, rheological and sensory characterization of Portuguese model cheeses manufactured from several milk sources. *Food Sci.Technol*. **44**, 2244–2252.

Reshetnik, L.A., Prokopeva, O.V. & Kochiev, N.K. 1997. Dietary and medical meaning of Jerusalem artichoke. *Nutrition Questions* **3**, 11–14.

Salhab, H.H. 1998. The application of ultrafiltration in the manufacture of cream cheese. *MSc. Thesis. Fac. Grad. Studies Res.* Edmonton, Alberta, Canada, 100 pp.

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On the potential of lupin protein concentrate made by enzymatic hydrolysis of carbohydrates in dairy-like applications

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Abstract. The aim of this research was to study the parameters of obtaining lupin concentrates by enzymatic hydrolysis of wholegrain lupin flour and application of these concentrates in the technology of high-protein dairy-like products. The following enzymes preparations were used: Celluclast BG, Cellulaza 100, Pentopan Mono BG and α-amylase. The usage of Cellulaza 100 and Pentopan Mono BG showed the highest protein amounts in the lupin concentrates under the test conditions. Three factors were studied to take effect on crude protein content in the product: temperature in the range 50–60°C; cellulase dosage between 0.54–1.62 units g^{-1}; ratio water: flour – 10 : 1, 15 : 1, 20 : 1. Optimum hydrolysis conditions for Cellulaza 100 were temperature of 55°C; ratio water: flour of 15 : 1; cellulase dosage of 1.08 units g^{-1}. Crude protein content in the final product increased on 12% compared with the original flour and on 8–9% compared to the lupin concentrate obtained without enzymes. Hydrolysis by multienzymatic compositions was tried as an alternative way of increasing the efficiency of the process. However hydrolysis by multienzymatic compositions was not yet found so efficient as hydrolysis by pure Cellulaza 100. The lupin protein concentrate was dispersed in water and mixed with skimmed milk to have total product protein content about 5%. The mixture was fermented by yogurt starter culture; consumer properties of final products were investigated. Fermented products supplement the diet with vegetable proteins, fats, carbohydrates and fiber, which have high biological value.

Key words: lupin protein concentrates, lupin whey, multienzymatic compositions, fermented products, analogues of dairy products.

INTRODUCTION

Limitation of resources of food, caused by both environmental and demographic factors, makes the scientists and manufacturers looking for the new ways to meet the needs in essential food nutrients. The main interest in lupin for foods is related to its high content of protein which is considered as a good source of lysine (El-Adawy et al., 2001). Typically, lupin seeds have a crude protein content of 31–42%, which is higher than the content of most other grain legumes (Pollard et al., 2002). Lupin already has many human consumption applications, such as bread making, pasta products, sausage substitutes, egg and milk replacers (Xu & Mohamed, 2003; Xu et al., 2006). Sweet lupin diet helps lowering whey cholesterol level (Chango et al., 1998). Whole lupin flour-enriched foods as well as isolated lupin protein and fiber fractions appear to have a cardioprotective action (Belski, 2012).

Yet the application of lupin products in food is limited, largely due to their 'green' and 'bean-like' flavor and antinutrirional substances. Fermentation of lupin

protein extracts using several lactic acid bacteria was conducted to reduce off-flavour formation in stored samples (Schindler et al., 2011). The main antinutritional substances in lupin are various alkaloids of the quinolizidine group. Hexane-defatted flakes of lupin (*Lupinus rautabilis*) were extracted under various conditions with alcohols to remove alkaloids (Blaicher et al., 1981). Production of protein isolates can overcome this problem too. Alkaloids are water-soluble and would be removed during preparation of the isolates (Lqari et al., 2001). The usage of some organic solvents improves lupin protein isolates quality (Bader et al., 2011).

Protein concentrates are considered to have greater than 50% protein and they are usually native flour (dehulled kernels) from which the carbohydrates (free sugars and oligosaccharides) and other soluble materials have been removed. Australian scientists have proposed the method of obtaining the protein preparation from lupin grains, which includes selection, treatment and fractionation of grains, their fragmentation, extraction of the protein, precipitation and drying (Sipsas, 2003). Muranyi et al. (2013) have studied two important techniques of protein isolation: the alkaline extraction with subsequent isoelectric precipitation and the salt-induced extraction followed by dilutive precipitation.

Plant proteins isolates and concentrates are used in the production of meat products, analogues of dairy products and combined foods. Lupin seeds and lupin seed protein isolates were used in the manufacture of fermented sausages (Papavergou et al., 1999). Soybeans are used as raw material to obtain soy yoghurt. It is hypoallergenic, so such products can be used in the diet of people suffering from milk protein intolerance (Osman & Razig, 2010; Vij et al., 2011). Soy-coconut yogurts were also studied (Kolapo & Olubamiwa, 2012). The milk-like product from *Lupinus campestris* was obtained by using an alkaline thermal treatment (Jimenez-Martınez et al., 2003).

Usage of enzymes is an alternative removal antinutritional substances method. Microbial hydrolytic enzymes are able to destroy many antinutritional components in plant raw material, which perform linking and protective function, such as phytin, cellulose, hemicellulose and lignin, and others (Ferket & Middleton, 1998). The improvement of carbohydrate extractability due to hydrolysis of polysaccharides allows producing a final product with higher protein content.

The most widespread, commercial enzyme products currently available for biomass hydrolysis are produced by submerged fermentation of the saprophytic mesophilic fungus *Trichoderma reesei* (Olsson & Ahring, 2007). The commercial enzyme preparation 'Celluclast' is a multiactive carbohydase for degradation of cellulose, cellobiose and higher polymers of glucose that could be used for improving malt quality (Grujic, 1998). Usage of Celluclast 1.5L for pectin extraction increases the pectin yield (Yuliarti et al., 2011). The degradation of biomaterial by cellulase is accompanied by the release of substrates for the action of other enzymes, particularly for xylanase and mannanase. The glyukuronoksilan and mannan are rapidly decomposed in a system with cellulase (Viikari et al., 1994). The application of arabinofuranosidase with xylanase leads to complete removal of xylan (Makkonen & Nakas, 2005).

The aim of this research was to study the parameters of obtaining lupin concentrates by enzymatic hydrolysis of wholegrain lupin flour and application of these concentrates in the technology of high-protein dairy-like products.

MATERIALS AND METHODS

Materials

Lupinus angustifolius wholegrain flour was provided by the All-Russian Scientific Research Institute of Lupin, Bryansk, with a crude protein content of 46%, crude fat – 7.1%, fiber – 4.0%.

Selection of enzyme preparations was carried out on the basis of carbohydrate composition of lupin seeds, which includes fiber, small share of starch (about 4%), hemicelluloses and pectin (in total 10%) (Kupcov & Takunov, 2006).

The following enzymes preparations were used:

- Celluclast BG – cellulase preparation made by submerged fermentation of the selected strain of fungus *Trichoderma reesei*, containing 3,500 endoglucanase units $gram^{-1}$. Preparation was provided by Novozymes, Denmark;
- Cellulaza 100 – cytolytic complex enzyme preparation derived from a mixed culture of fungi *Aspergillus foetidus* and *Trichoderma viride*, containing 540 cellulase units $gram^{-1}$, Sibbiofarm, Russia;
- Pentopan Mono BG – xylanase preparation from fungi *Aspergillus oryzae*, containing 2,500 fungal xylanase units $gram^{-1}$, Novozymes, Denmark;
- α-amylase, containing 950 fungal amylase units $gram^{-1}$. Preparation was provided by State scientific institution All-Russia Scientific Research Institute of Fats of Russian Academy of Agricultural Sciences, Russia.

The yogurt starter culture of *Streptococcus salivarius ssp. thermophilus and Lactobacillus delbrueckii ssp. bulgaricus* were provided by the State scientific institution All-Russia Scientific Research Institute of Fats of Russian Academy of Agricultural Sciences, Russia.

Preparation of the lupin concentrate

The used method is close to the one for obtaining the soy concentrate in the acidic medium (Sair, 1959). The full-fat lupin flour was diluted with water in a ratio of 1 : 10. The resulting solution was adjusted to pH 4.5 by adding 5% HCl to achieve protein isoelectric point. The following process was carried out in a thermostatic vessel with a magnetic stirrer (Khalil et al., 2006). Different variants of enzyme preparations were added after the suspension reached a temperature of 50ºC. The mixture was maintained for 40 min.

The mechanical separation of phases was performed by centrifugation at 4000 × g for 30 min. The sediment obtained after centrifugation was the final product (the lupin concentrate). The sediment contained protein and fat fractions as the flour used had a fat content of 7.1%.

The sediment protein content was analyzed after centrifugation. Total content of protein, water-soluble carbohydrates and their component composition in the lupin whey (supernatant) were analyzed.

Preparation of the fermented products

For making the non-dairy vegetable yoghurt analog lupin protein concentrate was dissolved in water to a dry matter content of 10%. The dispersion was neutralized with alkali to pH 6.8–6.9, homogenized at 60–65ºC, pasteurized at 92ºC for 3 min and

cooled to a temperature of fermentation. Sucrose was added in an amount of 1% to increase the quantity of nutrients for a starter culture. Fermentation was carried out to achieve the required pH values of 3.8–4.2.

The combined dairy vegetable product was made by adding skim milk instead of sucrose to the pasteurized dispersion in ratio of dairy and lupin proteins 50 : 50. Total protein content of the mixture was about 5%. Fermentation was held to pH 4.4–4.5.

For all products the yogurt starter culture was used in an amount of 5% of the system mass. The temperature of fermentation was 42 ± 2ºC.

Measurements

Water content in the lupin protein concentrate was determined by the gravimetric method (AOAC, 1998). The content of crude protein was determined by Kjeldahl method on automated analyzer Kjeltec Auto (Tecator, Sweden) according to standard protocol of manufacturer. Crude protein content was estimated using a conversion factor 6.25 from total nitrogen.

The content of crude fat was determined by the Soxhlet method on automated analyzer SER 148 (VELP Scientifica, Italy) according to standard protocol of manufacturer. The ceramic fiber filter method was used to determine the crude fiber (AOAC, 1980).

Analysis of the total content of water-soluble carbohydrates was conducted by Bertrand method (Bertrand & Thomas, 1910). Changes in pH were measured with Orion 920A pH-meter (Russia).

Component analysis of mono- and disaccharides was conducted by HPLC 'Stayer' (Akvilon, Russia) with refractometric detector, the column 'Luna NH_2 5μ', (Phenomenex, USA). The mobile phase consisted of acetonitrile and water in volume ratio of 77 : 23. 'Stayer' HPLC system with spectrophotometric detector and column 'Luna C18' (Phenomenex, USA) was used for analysis of the organic acids in whey, which was obtained by centrifugation of the fermented products samples at 4,000 × g for 30 min. Solution of 0.1% orthophosphoric acid in distilled water was used as a mobile phase.

Statistical evaluation of the data

All experiments were performed with at least three replicates; data was processed by methods of mathematical statistics at theoretical frequency 0.95. Statistical processing of data was carried out using computer programs Microsoft Office Excel 2010 and Mathcad 15.0.

RESULTS AND DISCUSSION

Effect of the enzyme preparations on the lupin concentrate

Enzymes with different substrate specificities were tried for the hydrolysis of lupin flour polysaccharides. Bioconversion efficiency was evaluated by the content of crude protein in the lupin concentrate and in the lupin whey (Table 1). The data was compared with the results for the negative control sample (the lupin concentrate obtained without enzymes).

Part of the water-soluble protein fraction is transferred to the whey during the lupin concentrate making; despite the system pH value of 4.5 is near the isoelectric

point. On average lupin concentrate loses 19% of protein with the lupin whey according to the mass balance. The usage of Cellulaza 100 and xylanase preparation leads to the highest protein amounts in the lupin concentrates under the test conditions. Cellulaza 100 was used for further optimization of the hydrolysis conditions.

Table 1. Crude protein and water content in the products of the lupin concentrate making, %

The name of the enzyme	Concentrate protein, on a dry basis	Concentrate water	Whey protein, on a dry basis
Control sample	50.4 ± 1.3	75.2 ± 1.1	28.0 ± 1.0
Cellulaza 100	53.3 ± 1.1	77.9 ± 0.5	34.0 ± 1.5
Celluclast BG	50.0 ± 1.1	79.2 ± 0.6	30.4 ± 1.0
Pentopan Mono	53.0 ± 1.2	78.6 ± 0.9	34.1 ± 1.4
α-amylase	48.2 ± 1.1	71.6 ± 0.4	29.4 ± 1.2

Optimization of the hydrolysis conditions

Optimum hydrolysis conditions were necessary for increasing the efficiency of the process. Three factors were studied to take effect on crude protein content in the product (Y, % to dry substance): temperature in the range of 50–60°C (Z_1); cellulase dosage of 0.54–1.62 u g^{-1} (Z_2); ratio water: flour – 10 : 1, 15 : 1, 20 : 1 (Z_3). The mixture was exposed to the hydrolysis for 40 min.

Optimal process parameters were obtained by means of rotatable plan of the second order and regression equation coefficients were found. The resulting response surfaces have the form of an elliptic paraboloid (Fig. 1).

The significance of the regression equation coefficients was determined by the Student's criterion. The adequacy of the regression equation was estimated by the *Fisher* test.

Optimum hydrolysis conditions for Cellulaza 100 were temperature of 55°C; ratio water: flour of 15 : 1; cellulase dosage of 1.08 units g^{-1}. Crude protein content in the final product under these conditions was 59.3 ± 1.1% on a dry basis. Crude protein content increased on 12–13% compared with the initial flour and on 8–9% compared to the lupin concentrate obtained without enzymes.

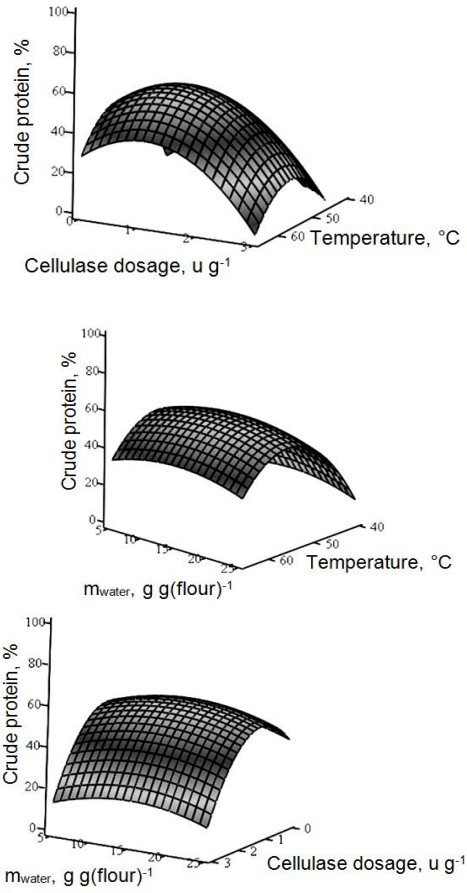

Figure 1. Dependence of crude protein content in the lupin concentrate on temperature, ratio water: flour and cellulase dosage.

Hydrolysis by multienzymatic compositions

Hydrolysis by multienzymatic compositions was tried as an alternative way of increasing the efficiency of the process. The degradation of non-starchy polysaccharides was conducted in the presence of Cellulaza 100 or Celluclast BG. An attempt was made to improve Cellulaza 100 hydrolysis efficiency with help of α-amylase. The synergetic effect is known between xylanase and cellulase. These enzymes act on cellulose, xylan and other hemicelluloses of lupin flour (Jeffries, 1996). So there was an attempt to raise the protein yield of the concentrates made with Celluclast BG with help of the xylanase preparation Pentopan Mono.

The compositions of the ferments included cellulases in the optimal dosage of 1.08 u g^{-1} and other enzymes in recommended or higher dosages. The ratio of enzymes in the compositions was calculated according to their declared activity (Table 2). Hydrolysis was carried out in the previously found optimal conditions for Cellulaza 100.

Table 2. Effect of multienzymatic compositions on the content of crude protein, fat and fiber in the lupin concentrates

No	Multienzymatic composition	Content in % on a dry basis		
		crude protein	crude fat	crude fiber
1	1.08 ± 0.02 u g^{-1} 'Cellulaza 100' & 0.7 ± 0.2 u g^{-1} α-amylase	54.9 ± 1.4	10 ± 1	5 ± 1
2	1.08 ± 0.02 u g^{-1} 'Celluclast BG' & 5 ± 1 u g^{-1} 'Pentopan Mono'	56.0 ± 1.1	10 ± 1	5 ± 1
3	1.08 ± 0.02 u g^{-1} 'Celluclast BG' & 25 ± 1 u g^{-1} 'Pentopan Mono'	51.9 ± 1.3	9 ± 1	5 ± 1

About 20% of cellulose was subjected to bioconversion to soluble carbohydrates according to the material balance. Multienzymatic composition based on cellulase 1.08 ± 0.02 u g^{-1} and xylanase 5 ± 1 u g^{-1} showed highest protein yeild results under the used conditions. However hydrolysis by multienzymatic compositions was not yet found so efficient as hydrolysis by pure Cellulaza 100. Moreover, increased content of Pentopan Mono resulted in reduced crude protein content in the lupin concentrate of the third sample. This may be due to the inhibition of the action of one enzyme preparation by hydrolysis products of other one. This issue requires further study.

Transition of carbohydrate and protein fractions in the lupin whey

The lupin whey of samples after hydrolysis with 1.08 ± 0.02 u g^{-1} 'Celluclast BG' & 5 ± 1 u g^{-1} 'Pentopan Mono' was analyzed as they showed highest protein yeild results under the used conditions for multienzimatic compositions. The resulting lupin whey contains some extractive substances from lupin seeds (organic acids, soluble carbohydrates and vitamins, other biologically active substances), molecular nitrogen compounds (amino acids, peptides, albumin fraction of proteins) and lipids, which were released from the initial substrate in the process of hydrolytic destruction of cellular structures (Table 3). The initial lupin flour and the resulting lupin whey had a dry substance content of 90% and 2%, respectively.

Table 3. Crude protein, mono- and disaccharides percentage of the initial lupin flour and resulting lupin whey

	% to dry substance	
	flour	whey
Crude protein	46.3 ± 1.1	30.0 ± 1.4
Mono- and disaccharides	3.4 ± 0.5	25.0 ± 0.3

The balance of material is shown in equation 1:

$$M_{flour} \cdot S_{flour} \cdot C_{flour} = M_{whey} \cdot S_{whey} \cdot C_{whey}, \text{ or } 100 \cdot 0{,}90 \cdot 0{,}034 \rightarrow 1280 \cdot 0{,}02 \cdot 0{,}25 \qquad (1)$$

where: M – mass of the product (g); S – solids (dry substance), share units; C – carbohydrates, share units.

Total content of mono- and disaccharides in the lupin whey according to the material balance has increased in 2 times in comparison with their content in the initial flour.

Mass fraction of total sugar (to invert sugar) in the whey was 0.5% (25% to dry substance). Non-starchy polysaccharides hydrolysis increases the concentration of soluble disaccharides (in particular, sucrose) and monosaccharides in the whey up to 17% and 3% to dry substance, respectively. High performance liquid chromatography of the lupin whey showed that the ratio sucrose: glucose: fructose is 10 : 1 : 1.

Transition of protein fraction to the whey was 18% of the amount contained in the flour. That corresponds to the data obtained for the samples hydrolysed with Cellulaza 100.

Thus, 20% of the hydrolyzed cellulose and some quantity of the hydrolyzed xylan increase yields of mono- and disaccharides in the whey twice but accompanied by the protein loss of 18%.

Application of the lupin concentrates in dairy-like products

Three fermented products were studied: the non-dairy vegetable yoghurt analog, the combined dairy vegetable product and the control product based on a skim cow milk. All received clots had homogeneous consistency with minor release of whey, sour-sweet taste and fruity smell. The usage of vegetable raw material in the combined product leads to dynamic reduction of pH in the first 6 h of fermentation comparing to the control product (Fig. 2). This gives the possibility to reduce the fermentation time.

The initial pH value of the lupin dispersion was 1.2 units lower than in the control sample. Buffer capacity of this dispersion was lower compared to the skim milk because of its plant origin.

Composition of organic acids in the whey obtained by centrifugation of the products samples characterizes the biochemical process of fermentation (Table 4).

Increase in the acidity of the combined product in comparison with the control product mainly depends on the malic acid production. This is due to the formation of by-products of homofermentative lactic acid fermentation, in particular malic acid. Organic acids are produced in varying degrees in the process of fermentation and storage of yoghurts (Fernandez-Garcia & McGregor, 1994). The course of lactic acid

fermentation can be different depending on environmental conditions. Malic, propionic and some other organic acids are formed on the Embden-Meyerhof-Parnas pathway.

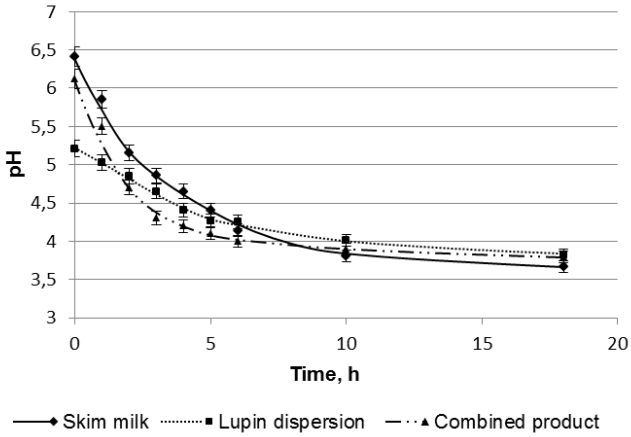

Figure 2. Dependence of active acidity on the fermentation time and the product base.

Table 4. Composition of organic acids in the whey samples

Whey sample	Content of lactic acid, g dm^{-3}	Content of malic acid, g dm^{-3}	The amount of lactic and malic acids, g dm^{-3}
Vegetable analog	3.93 ± 0.19	0.17 ± 0.03	4.1
Combined product	6.7 ± 1.3	1.9 ± 0.4	8.6
Control product	6.9 ± 1.2	–	6.9

Table 5. Macronutrients and energy value of the fermented products

Parameter	Vegetable yoghurt analog	Combined product	Control product
Protein, %	4.8 ± 0.2	4.8 ± 0.2	5.0
Fat, %	0.8 ± 0.1	0.6 ± 0.2	1.5
Fiber, %	0.4 ± 0.1	0.2 ± 0.1	–
Energy value, kcal (per 100 g)	61.3	67.4	75.1

The fermented products supplement the diet with vegetable proteins, fats, carbohydrates and fiber, which are necessary for the proper functioning of the gastrointestinal tract and have high biological value (Table 5).

The vegetable yoghurt analog and the combined product can be classified as low-calorie dietary foods. Fiber in the composition of these products would have a positive effect on intestinal motility. Sensory analysis of the products with hydrolyzed lupine flour revealed no ‘green’ and ‘bean-like’ flavors.

CONCLUSIONS

Enzymatic treatment of the lupin flour is an effective method for increasing the protein content in the concentrate production. Hydrolysis of the lupin flour with Cellulaza 100 increased crude protein content in the lupin concentrate on 12–13% compared with the initial flour and on 8–9% compared to the lupin concentrate obtained without enzymes. This led to crude protein content in the concentrate of 59.3 ± 1.1% on a dry basis. Average protein loss from moving in the whey amounted 18–19%. Improving efficiency of hydrolysis using a combination of enzyme preparations still not yielded the expected results, and requires further study.

The formation of malic acid during lactic acid fermentation of products containing lupin concentrates gives the possibility to reduce the fermentation time. The received products have homogeneous consistency, sour-sweet taste and fruity smell without negative flavor. They can be classified as low-calorie dietary foods with energy value less than 70 kcal per 100 g.

The proposed ways of making fermented products allow reducing the cost of vegetable yoghurt analogue in 1.7 times, the combined product in 1.3 times compared with the traditional product in Russia. Implementation of these products can help diversifying production due to the necessity of compensation of technological risks and of using new food sources.

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REFERENCES

AOAC. 1998. *Official Method of Analysis*. Association of Official Analytical Chemists (16th Edn), Washington DC, USA.

AOAC. 1980. *Official Method 962.09.* Fiber (Crude) in Animal Feed and Pet Food. Association of Official Analytical Chemists (13th Edn), William Horwit (Ed.) Washington DC.

Bader, S., Oviedo, J.P., Pickardt, C. & Eisner, P. 2011. Influence of different organic solvents on the functional and sensory properties of lupin (Lupinus angustifolius L.) proteins. *LWT – Food Sci.Techn.* **44**, 1396–1404.

Belski, R. 2012. Fiber, Protein, and Lupin-Enriched Foods: Role for Improving Cardiovascular Health. *Adv. Food Nutr. Res.* **66**, 147–215.

Bertrand., G. & Thomas, P. 1910. *Guide pour les manipulations de chimie biologie*, Dunod et Pinat, Paris, 468 pp.

Blaicher, F.M., Nolte, R. & Mukherjee, K.D. 1981. Lupin Protein Concentrates by Extraction with Aqueous Alcohols. *J Am. Oil Chem. Soc.* **58**(7), 761–765.

Chango, A., Villaume, C., Bau, H.M., Schwertz, A., Nicolas, J.P. & Mejean, L. 1998. Effects of Casein, Sweet White Lupin and Sweet Yellow Lupin Diet on Cholesterol Metabolism in Rats. *J. Sci. Food Agric.* **76**, 301–309.

El-Adawy, T.A., Rahma, E.H., El-Bedawey, A.A., & Gafar, A.F. 2001. Nutritional potential and functional properties of sweet and bitter lupin seed protein isolates. *Food Chem.* **74**(4), 455–462.

Ferket, P.R. & Middleton, T. 1998. Antinutrients in poultry feeds. *Proc. World Poult. Sci. Assoc. 10th Europe Poultry Conference.* Jerusalem, Israel, pp. 43–52.

Fernandez-Garcia, E. & McGregor, J.U. 1994. Determination of organic acids during the fermentation and cold storage of yogurt. *J.Dairy Sci.* **77**, 2934–2939.

Grujic, O. 1998. Application of a commercial enzyme preparation in the barley malting process. *J. Inst. Brew.* **104**, 249–253.

Jeffries, T. 1996. Biochemistry and genetics of microbial xylanases. *J. Biotechnol.* 7, 337–342.

Jimenez-Martınez, C., Hernandez-Sanchez, H. and Davila-Ortiz, G. 2003. Production of a yogurt-like product from Lupinus campestris seeds. *J. Sci. Food Agr.* **83**(0022–5142), 515–522.

Khalil, A.A., Mohamed, S.S., Taha, F.S. & Karlsson, E.N. 2006. Production of functional protein hydrolysates from Egyptian breeds of soybean and lupin seeds. *Afr. J. Biotechnol.* **5**(10), 907–916.

Kolapo, A.L., & Olubamiwa, A.O. 2012. Effect of Different Concentrations of Coconut Milk on the Chemical and Sensory Properties of Soy-coconut Milk Based Yoghurt. *Food and Public Health* **2**(4), 85–91.

Kupcov, N.S. & Takunov, I.P. 2006. *Lupin genetics, breeding, heterogeneous crops.* Klincovskaja gorodskaja tipografija, Brjansk, 576 pp. (in Russian).

Lqari, H., Vioque, J., Pedroche, J. & Millán, F. 2002. Lupinus angustifolius protein isolates: chemical composition, functional properties and protein characterization. *Food Chem.* **76**, 349–356.

Makkonen, H. & Nakas, J. 2005. Use of xylanase and arabinofuranosidase for arabinose removal from unbleached kraft pulp. *Biotechnol. Lett.* **27**, 1675–1679.

Muranyi, I.S., Otto, C., Pickardt, C., Koehler, P. & Schweiggert-Weisz, U. 2013. Microscopic characterisation and composition of proteins from lupin seed (Lupinus angustifolius L.) as affected by the isolation procedure. *Food Res. Int.* **54**, 1419–1429.

Olsson, L. & Ahring, B.K. 2007. Biofuels. Springer, Berlin, 368 pp.

Osman, M.M.D., & Razig, K.A.A. 2010. Quality Attributes of Soy-yoghurt During Storage Period. *Pakistan J. Nutr.* **9**(11), 1088–1093.

Papavergou, E.J., Bloukas, J.G. & Doxastakis, G. 1999. Effect of lupin seed proteins on quality characteristics of fermented sausages. *Meat Sci.* **52**, 421–427.

Pollard, N.J., Stoddard, F.L., Popineau, Y., Wrigley, C.W. & MacRitchie, F. 2002. Lupin Flours as Additives: Dough Mixing, Breadmaking, Emulsifying, and Foaming. *Cereal Chem.* 79 (5), 662–669.

Sair, L. 1959. *Proteinaceous soy composition and method of preparing*. US Patent 2,881,076.

Schindler, S., Wittig, M., Zelena, K., Krings, U., Bez, J., Eisner, P. & Berger, R.G. 2011. Lactic fermentation to improve the aroma of protein extracts of sweet lupin (Lupinus angustifolius). *Food Chem.* **128**, 330–337.

Sipsas, S. 2003. Protein concentrates and isolates. *Seeding a Future for Grains in Aquaculture Feeds* **28**, 7–9.

Viikari, L., Kantelinen, A., Buchert, J. & Puls, J. 1994. Enzymatic accessibility of xylans in lignocellulosic materials. *Appl. Microbiol. Biotechnol.* **41**, 124–129.

Vij, S., Hati, S. & Yadav, D. 2011. Biofunctionality of Probiotic Soy Yoghurt. *Food Nutr. Sci.* **2**, 502–509.

Xu, J., & Mohamed, A. A. 2003. Thermal and rheological properties of Lupinus albus flour. *J Am. Oil Chem. Soc.* **80**(8), 763–766.

Xu, J., Mohamed, A.A., Hojilla-Evangelista, M.P., & Sessa, D.J. 2006. Viscoelastic properties of lupin proteins produced by ultrafiltration-diafiltration. *J Am. Oil Chem. Soc.* **83**(6), 553–558.

Yuliarti, O., Matia-Merino, L., Goh, K.K., Mawson, J.A. & Brennan, C.S. 2011. Effect of Celluclast 1.5L on the Physicochemical Characterization of Gold Kiwifruit Pectin. *Int. J. Mol. Sci.***12**, 6407–6417.

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Quantitation of vitamins A and E in raw sheep milk during lactation period

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Abstract. In this article, the influence of breed and lactation stage on vitamin A and E content in raw sheep milk was studied. The milk of the East Friesian, Romanov and Lacaune sheep breeds was included in the study. The samples were taken once a month throughout lactation. The total average content of vitamin A in raw milk of all sheep breeds during lactation was 0.76 ± 0.19 mg kg^{-1} of milk and the total average content of vitamin E was 2.86 ± 0.99 mg kg^{1} of milk. The content of vitamin A and E during lactation varied in respect to the breed type. The highest average content of vitamin A and E was detected in the Romanov sheep:1.01 ± 0.19 mg kg^{-1} of milk for vitamin A and 4.26 ± 1.90 mg kg^{-1} for vitamin E. The lowest average value of vitamin A was found in the milk of the East Friesian sheep (0.56 ± 0.10 mg kg^{-1}). This milk showed also the lowest content of vitamin E 2.11 ± 0.53 mg kg^{-1}. The highest content of vitamins was found in summer and at the end of lactation, which is in September, and the lowest values appeared in early lactation, which is in April.

Key words: Lactation, sheep milk, sheep breed, vitamin A, vitamin E.

INTRODUCTION

The production of sheep milk has been increasing in the Czech Republic in recent years. People are becoming more and more aware of the milk and dairy products of these small ruminants. Due to the increasingly expanding network of farmers' markets, it is much easier to get hold of these products. The East Friesian and Romanov sheep are the most widely bred breeds in the Czech Republic. The Lacaune sheep have gained popularity only recently in the Czech Republic. Sheep milk is much easier to digest than cow milk. Its fat is dispersed in smaller fat beads (Jandal, 1996). In addition, due to the better digestibility of sheep milk proteins, sheep milk is an important constituent of the diet of people suffering from allergies. Milk contains relatively low amounts of vitamin A and E; however, due to its frequent consumption in various forms, it represents an important dietary resource, taking into account the higher average fat content in sheep milk (7%) (Raynal-Ljutovac et al., 2008). Thus, a higher expression of lipophilic vitamins in sheep milk when compared to cow and goat milk is to be expected.

Vitamin A plays a role in the biochemical pathways related to visual perception, affects the growth, differentiation and maturation of gametes, and is important for fetal development, growth, and bone development (Debier et al., 2005). It plays a role in the synthesis of proteins, nucleic acids, and lipoproteins. Vitamin A is also an effective antioxidant. Vitamin deficiency is associated with vision disturbances (night blindness), inhibition of growth and deformities of bone and reproductive organs. High doses of vitamin A result in increased hepatic reserve. In pregnant women, it may have teratogenic effects (Miller et al., 1998). The recommended daily dose for an adult is in the range of 0.8 mg (2,600 IU) – 1 mg (3,300 IU) and for children from 0.4 to 0.6 mg (1,300–2,000 UI) (Capito and Calleja, 2006).

Vitamin E is a very important antioxidant. It has an important function in protecting the body against free oxygen radicals, which can lead to DNA damage. It is also a factor that slows down the ageing of the body and plays a role in the prevention of cardiovascular diseases and cancer (Eitenmiller & Junsoo, 2004). Vitamin E is present in food, being dissolved in fats, and is released and subsequently absorbed during their cleavage in the intestine. The recommended daily dose of vitamin E is from 10 to 15 mg for adults, this value is around from 5 to 8 mg for children (Monsen, 2000). Vitamin E deficiency is often associated with disorders of fat absorption or distribution or cystic fibrosis (Pekmezci, 2011).

The content of vitamins in raw milk is influenced by many factors. These include animal species, breed, stage of lactation, and individual health status. According to Zervas & Tsiplakou (2011), another important aspect is the nutrition of the animal and the specific character of farming.

There are not many studies, which focus on the investigation of vitamin content in raw sheep milk in the Czech Republic. The aim of our study was to determine the content of fat-soluble vitamins A and E in the milk of sheep from private farms in the Czech Republic and to assess the effect of lactation period on the content of these vitamins.

MATERIALS AND METHODS

Experimental material

Pooled milk samples of the East Friesian sheep (a herd of 380 head), the Romanov sheep (a herd of 130 head), and Lacaune sheep (a herd of 85 head) were collected throughout the lactation from April to September on private farms once a month, in cooperation with the Dairy Research Institute. The milk samples were taken first 20 days after parturition.

The ration of all breeds was based on full-day pasture *ad libitum*, hay, and silage. Mineral licks have been used in all cases as a dietary supplement: MILLAPFOS, BIOSAXON for the Romanov sheep, RUMIHERB, NATURMIX for the East Friesian sheep, and SANO for the Lacaune sheep. A mixture of pressed grains was also supplemented.

The chemicals. Determination of vitamins

For the preparation of the analytical samples, we used the following standards and chemicals: DL-α-tocopherol, 98.2% (CALBIOCHEM, Canada), tocopherol set (CALBIOCHEM, Canada), retinol, > 99% (Sigma-Aldrich, Germany), pyrocatechol, > 99.5% (Sigma-Aldrich, Germany), potassium hydroxide, min. 85% (Lachema, Czech Republic), methanol, p.a., content 99.5% (Lachner, Czech Republic), hexane, clean min. 95.0%, Penta, Czech Republic, methanol, super gradient, content min. 99.9% (Lachner, Czech Republic), and treated distilled water (Milipore, France).

Measurement of vitamin E and A content in milk samples

Vitamin E (or the individual tocopherols (T) and tocotrienols (TKT)) and vitamin A were determined by high performance liquid chromatography with fluorescence and spectrophotometric detection, respectively.

Approx. 1 g of homogenized sample was weighed in a plastic tube with a lid. 200 ml of methanol pyrocatechol (0.2 g ml^{-1}) was added, then 5 ml 1M KOH, and the mixture was vortexed for 20 seconds. Subsequently, the sample was saponified for 10 minutes on ultrasound. Then the mixture was vortexed again for 20 seconds. Then, 5 ml of hexane and 1 ml of distilled water were added to the mixture. The mixture was vortexed for 1 minute. Subsequently, 3 ml were taken from the upper hexane layer and evaporated on a rotary evaporator until dry. The residue was dissolved in 0.5 ml of methanol and an aliquot was transferred through a nylon filter into a 1 ml Eppendorf tube, which was placed in the freezer (-20ºC) for 30 minutes. Subsequently, the sample was centrifuged for 2 minutes (by 14.4 rpm) and drained off into a dark vial. For the analytic extension we used the chromatographic system Ultimate 3,000 (Dionex, USA), consisting of a quaternary high-pressure pump, an autosampler, a column thermostat, a fluorescence detector and a diode array detector. We employed an analytical column with a precolumn packed with Develosil 5µm RPAQUEOUS (250 × 4.6 mm); Develosil 5µm C30 UG-100A (10 × 4 mm) (Phenomenex, USA), which allows the separation of all forms of tocopherols and tocotrienols (Fig. 1).

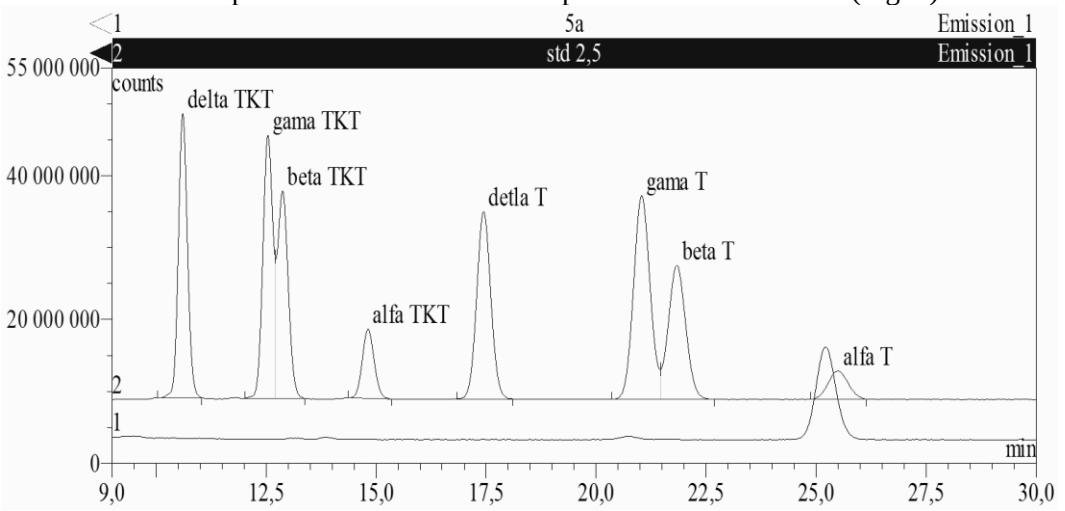

Figure 1. Chromatogram of vitamin E (compared to the standard): 2 – standard, 1 – sample.

A mixture of MeOH: deionized water (97 : 3, v/v) was used as the mobile phase; flow rate of 1 ml per minute. Column temperature was 30°C and 10 ml sample injection was applied. For the detection of tocol wavelengths we selected: excitation 292 nm and emission at 330 nm. Vitamin A was determined by spectrophotometric detection at λ = 325 nm (Fig. 2).

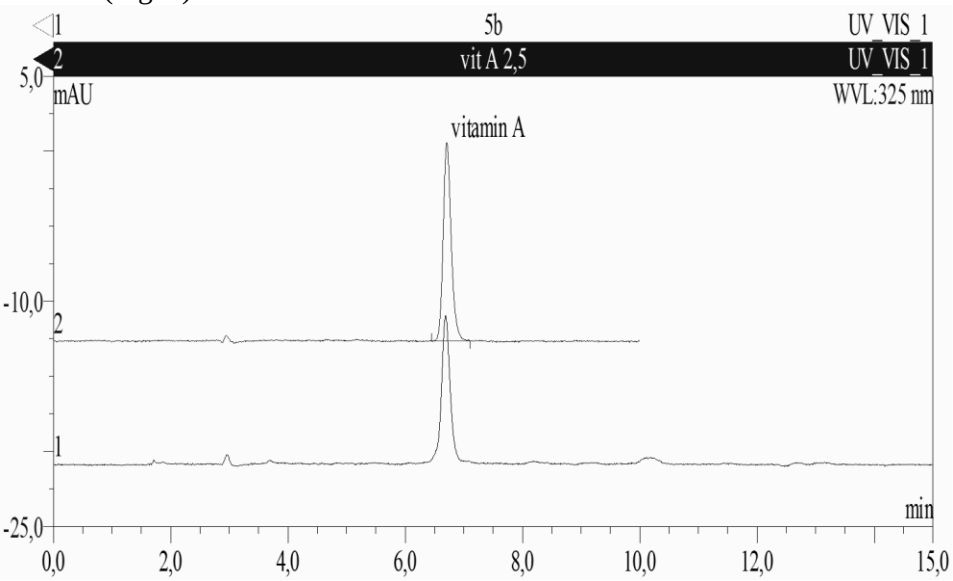

Figure 2. Chromatogram of vitamin A (compared to the standard): 2 – standard, 1 – sample.

The content of analytes in the samples was evaluated by external calibration. Calibration curve of all tocols and vitamin A was linear in the range from 0.05 to 10 μg ml^{-1}. The detection limit for each tocol, expressed as a ratio of three times the value of the signal-to-noise ratio, was as follows: δ-tocotrienols and δ-tocopherol 0.01 μg ml^{-1}, β-tocotrienol, γ-tocotrienol, β-tocopherol and γ-tocopherol 0.025 μ ml^{-1}, α-tocotrienol and α-tocopherol 0.05 μ ml^{-1}, vitamin A 0.025 μ ml^{-1}. The results were processed with Chromeleon and MS Excel. The results were expressed as m kg^{-1} of milk. Statistical analysis was done in *Statistica* Version 9. The measured values were processed by the analysis of variance method (*ANOVA*), using *post-hoc Tukey's* tests for more detailed evaluation.

RESULTS AND DISCUSSION

The content of vitamin A and E was detected in raw milk of 3 sheep breeds – the East Friesian, the Romanov and the Lacaune. The average values of vitamin A and E in the milk samples of individual breeds are presented in Table 1. The total average content of vitamin A in raw milk of all sheep breeds during lactation was 0.7–0.1 m kg^{-1} of milk and the total average content of vitamin E was 2.8–0.9 m kg^{-1}. The correlation between the content of vitamin A and vitamin E was found (R^2 = 0.62, $P < 0.05$).

The results show significant differences in the content of vitamins A and E in relation to the breed of sheep. The highest ($P < 0.05$) average content of both, vitamins A and E, was found in the milk of the Romanov sheep (1.01 ± 0.19 mg kg^{-1} and 4.26 mg $kg^{-1} \pm 1.90$, respectively) when compared with two other breeds. The average content of vitamin E in the milk of the Lacaune sheep vs. milk of the East Friesian breed was not statistically different while the difference between these two breeds was found for vitamin A in milk (Table 1.) Raynal-Ljutovac et al (2008) found the content of vitamin E in sheep milk 1.1 mg kg^{-1}. The content of vitamin A is according to this author 0.8 mg kg^{-1}. Park et al (2007) gives the value of vitamin A content 0.44 mg kg^{-1} (146 UI). Thus, values of vitamin A content determined in this study correspond to values published in Czech and foreign studies, while the content of vitamin E was higher than most of the published values related to sheep milk.

Table 1. The average content and standard deviation of vitamins A and E in sheep milk

	East Friesian	Lacaune	Romanov
Vitamin A	0.56 ± 0.10^{a}	0.70 ± 0.11^{b}	1.01 ± 0.21^{c}
Vitamin E	2.11 ± 0.53^{a}	2.21 ± 0.84^{a}	4.26 ± 1.91^{b}

The values in the same line marked with different letters (a–d) differ significantly ($P \leq 0.05$)

The amount of vitamins in sheep milk varies during lactation. The lowest measured value of vitamin E (0.15 mg kg^{-1}) among all the breeds was found in the milk of the Romanov sheep in April. The highest value was found in September (6.70 mg kg^{-1}) in the milk of the Romanov sheep, too. The content of vitamin E in the milk of the Lacaune sheep and the East Friesian sheep had a similar time-course to the milk of the Romanov sheep but the values were lower through all the lactation stages. The lowest content of vitamin E among all the breeds was found at the beginning of the lactation period, which is in April. The average value was 0.59 ± 0.39 mg kg^{-1}. The highest average value of vitamin E among all the breeds was recorded at the end of lactation, which is in September ($4.4 \pm$ mg kg^{-1}). In other months, the content of vitamin E was not statistically different from each other. It appears that the content of vitamin E in raw sheep milk rises from April to September, and thus increases during lactation (Fig. 3).

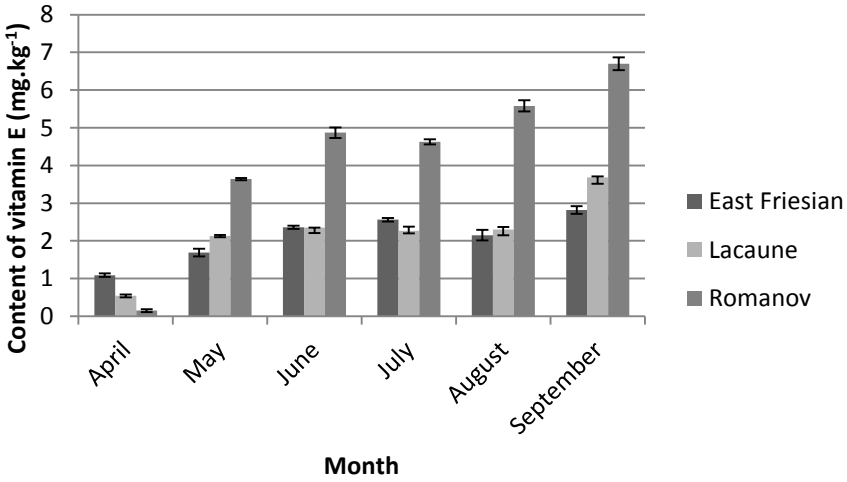

Figure 3. Content of vitamin E during lactation.

The values of vitamin A during lactation are recorded in Fig. 4. The lowest value of vitamin A (mg kg^{-1}) between all the monitored breeds was found in the milk of the East Friesian sheep in August. The highest content 1.25 mg kg^{-1} was recorded in September in the milk of the Romanov sheep. The values of vitamin A had a very similar time-course to that of vitamin E.

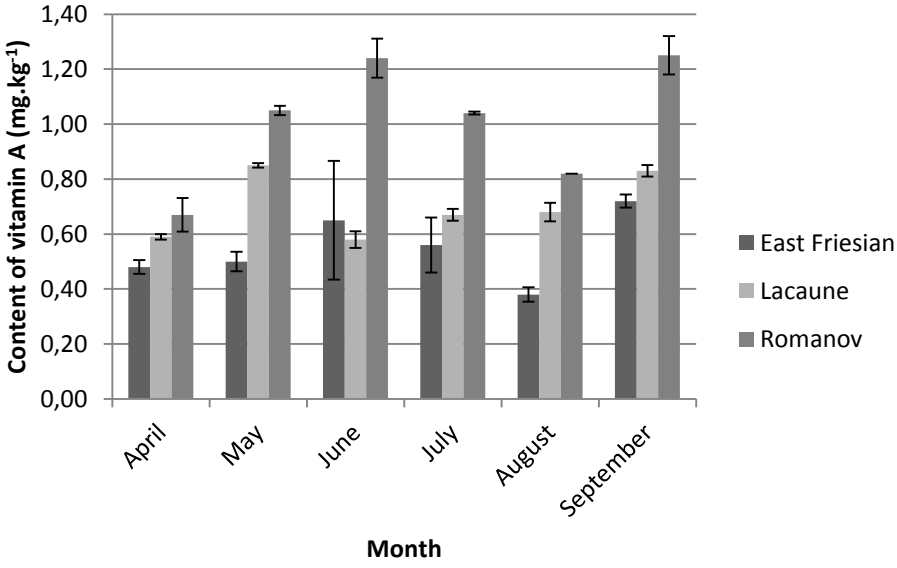

Figure 4. Content of vitamin A during lactation.

The content of vitamin A in milk increases from the beginning of the lactation period till June, then decreases slightly and takes the highest values in September. Statistically highest average content of vitamin A 0.93 mg kg^{-1} was determined in September, the lowest average content 0.58 ± 0.08 mg kg^{-1} in April. The values measured in May, June and July were not statistically different.

It can be presumed that fluctuations in the above mentioned vitamin levels during lactation are associated with warm weather in the summer months and also with changes in the fat content of sheep milk during lactation.

The content of both vitamins in sheep milk experimentally determined in this study was higher than the content of both vitamins in cow milk given in literature. The difference is more striking for vitamin E than for vitamin A (Table 2).

Table 2. Comparison of sheep and cow milk (average content)

	Sheep milk	Cow milk
Vitamin A	0.76 ± 0.19 mg kg^{-1}	0.30–1.00 mg kg^{-1}*
Vitamin E	2.86 ± 0.99 mg kg^{-1}	0.20–1.20 mg kg^{-1}*

*Fox & McSweeney (1998)

CONCLUSION

There were found significant differences in the content of vitamin A and E between three sheep breeds. The highest content of both, vitamins A and E, was found in the milk of the Romanov sheep. The milk content of both vitamins differed in respect to the lactation period as well. The highest content was found in September and the lowest in April. A strong correlation was found between the average content of the two examined vitamins over the entire monitored period. Sheep milk contains a higher amount of both vitamins compared to cow milk.

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REFERENCES

Capita R. & Caleja C.A. (2006). Evaluation of vitamin and mineral intakes and impact of snack foods on Spanish adults. *Nutr Res.* **26**, 255–265.

Debier, C., Pottier, J., Gofee, C.H. & Larondelle, Y. (2005). Present knowledge and unexpected behaviours of vitamins A and E in colostrum and milk. *Livest Prod Sci*. **98**, 135–147.

Eitenmiller, R. & Junsoo, L. 2004. *Vitamin E – Food Chemistry, Composition and Analysis*. Food Science and technology, Marcel Dekker, New York, 530 pp.

Fox, P.F. & Mcsweeney, P.L.H. 1998. *Dairy Chemistry and Biochemistry***.** Blackie Academic & Professional, an imprint of Chapman & Hall, London. 487 pp.

Jandal, J.P. (1996). Comparative aspects of goat and sheep milk. *Small Ruminant Res*. **22**, 177–185.

Miller, R.K., Hendrikx A.G., Mills, J.L., Hummler, H. & Wiegand, U.W. (1998). Perinconcepti onal vitamin A use: How much is teratogenic? *Reprod Toxicol.* **12**, 75–88.

Monsen, E.R. (2000). Dietary Reference Intakes for The Antioxidant Nutrients: Vitamin C, Vitamin E, Selenium, and Carotenoids. *J Am Diet Assoc*. **6**, 637–640.

Öste, R., Jägerstad, M., Anderson, I. 1997. Vitamins in milk and milk products**.** In Fox, P.F. (ed.): *Advanced Dairy Chemistry*, Vol. 3 Lactose, water, salts and vitamins. 2nd ed., Chapman & Hall, London. 536 pp.

Park, Y.W., Juáres, M., Ramos, M. & Haenlein, G.F.V. (2007). Physico-chemical characteristics of goat and sheep milk. *Small Ruminant Res.* **68**, 88–113.

Pekmezci, J. (2011): Vitamin E & Immunity. Vitamins and Hormones. **86**, 179–215.

Raynal-Ljutovac, K., Lagriffoul, G., Paccard, P., Guillet, I. & Chilliard, Y. (2008). Composition of goat and sheep milk products: An update. *Small Ruminant Res*. **79**, 57–72.

Zervas, G. & Tsiplakou, E. (2011). The effect of feeding system on the characteristic of product of small ruminants. *Small Ruminant Res.* **101**, 140–149.

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The effect of modified atmosphere storage on the postharvest quality of the raspberry 'Polka'

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Abstract. The aim of the experiment was to determine the effect of a passive modified atmosphere package (MAP) (30 μm LDPE bag (Estiko, Estonia), the Xtend® raspberry bag (Stepac, Israel) and an active MAP (30 μm LDPE bag, flushed with a gas mixture containing 10% O_2 and 15% CO_2) on the postharvest quality of the raspberry 'Polka'. Raspberries stored in macroperforated punnets (normal atmosphere – NA) served as the control. The raspberries were initially stored for 3 days at 1.6°C, then a half of the bags were moved into simulated retail conditions (6ºC) and a half remained at 1.6°C for another 24 hours.
The raspberry weight, O_2 and CO_2 content of the packages were measured daily. The fruit dry matter (DM) and soluble solids content (SSC), titratable acidity (TA), ascorbic acid content (AAC), and total anthocyanins (ACY) were determined at harvest and the total antioxidant activity (TAA) and rotting (weight of rotten berries) were determined after storage. Neither the active nor passive MAP suppressed rotting significantly. The raspberries stored at 1.6°C, had the best quality in passively modified LDPE bags, since the fruit had higher SSC and TA and lower ACY content compared to the control. After the simulated retail conditions, the Xtend® bags turned out to be the most suitable, since the fruit had the lowest ACY content (the fruit did not become too dark), but the highest TAA.

Key words: *Rubus idaeus*, soluble solids, titratable acids, ascorbic acid, anthocyanins, total antioxidant activity.

INTRODUCTION

Growing primocane raspberry cultivars is a good opportunity for berry growers in Estonia and other Nordic countries to prolong the fresh raspberry season. Also, pesticide need is reduced in primocane raspberries, since the oviposition period of the dominant pest, the raspberry beetle (*Byturus tomentosus* De Geer), does not coincide with the flowering period of the primocane cultivars (Vetek & Penzes, 2008). Also, frost damage during the winter does not affect the yield of primocane raspberries as much as it does summer fruiting raspberry cultivars.

The main risk in Estonia for cultivating primocane raspberry is the cold climate, since early autumn frosts and low temperatures in September may hinder raspberry ripening. The Polish primocane raspberry 'Polka' has been introduced as a self-supporting, semi vigorous, early autumn bearing, and high yielding cultivar (Danek,

2002). The first commercial plantations with the raspberry 'Polka' have been established in Estonia. Besides the local market, Finland is the main destination for Estonian berries and the producers are looking for cultivars, which would stand long distance transportation. Danek (2002) has stated that the fresh fruit of 'Polka' are firm and cohesive with tight skin. This statement makes it possible to hypothesize that this cultivar could have good storage potential. However, to our knowledge, postharvest experiment results with 'Polka' raspberries have not been published yet.

The aim of the current research was to determine the effect of normal and modified atmosphere storage on the postharvest quality of the raspberry 'Polka', considering both the external quality and the nutritional value of the fruit.

MATERIALS AND METHODS

The 'Polka' raspberries were grown in a commercial plantation in South Estonia (NL 58º15'33''; EL 26º35'33''), where brown pseudopodzolic soils dominate. The plantation was 3 years old and was established using black polyethylene mulch and drip irrigation. The plant to plant spacing was 0.5 m and the distance between the rows was 3 m. Weather conditions in 2008 were not favourable for raspberries, since the summer was very rainy. The fruit development and ripening period in August was extremely wet, since 216 mm rained, which was almost three times more than the average (79 mm).

The raspberries were harvested at commercial maturity on September 14. The fruit with uniform size and colour and free from defects were picked directly into 250g macroperforated plastic punnets, transported to the university, cooled down to 1.6°C and packed as follows:

1) 250g macroperforated plastic punnets covered with a lid (control);
2) Xtend® raspberry bag (Stepac, Israel);
3) passive modified 30 μm low density polyethylene (LDPE) bag (Estiko, Estonia);
4) actively modified 30 μm LDPE bag, flushed with a gas mixture containing 10% O_2 and 15% CO_2).

One treatment consisted of six replicate bags (four punnets in one bag). The raspberries were stored for 4 days: on the 3rd day, a half of the bags were moved into simulated retail conditions (6ºC) and a half remained at 1.6°C. The storage room relative humidity ranged from 96 to 98%.

The raspberries were weighed and the O_2 and CO_2 contents measured from the packages every day with a hand-held gas analyser OXYBABY V (WITT-Gasetechnik GmbH & Co KG, Germany). The fruit dry matter (DM) and soluble solids content (SSC), titratable acidity (TA), ascorbic acid content (AAC), and total anthocyanins (ACY) were determined at harvest and at the end of storage. Total antioxidant activity (TAA) and rotting (weight of berries with rots) were determined after storage. A fruit was considered rotten if even one of the drupelets was infected.

For determination of the AAC, ACY and TAA, ten randomly chosen fruit from each treatment were weighed into a titration vessel separately for each analysis and extraction solution was added immediately to avoid breakdown of the easily oxidised compounds in the air. For the AAC determination, the fruit were crushed quickly with

a homogenizator and titrated with dichlorophenolindophenol; also, voltamperometric indication was used (method M569/570 (www.mt.com)). TA was determined by titration to pH 8.2 with 0.1 NaOH. The Titrator Mettler Toledo DL50 with the autosampler Rondolino was used for titration of the AAC and TA. The SSC (%) was measured using the digital refractometer PAL-1 (ATAGO CO., Ltd., Japan). The content of total anthocyanins was estimated by a pH differential method (Cheng & Breen, 1991). Total anthocyanins were calculated as cyanidin-3-glycoside, one of the major anthocyanins in raspberries (Boyles & Wrolstad, 1993). The TAA was determined using the 1.1-diphenyl-2-picrylhydrazyl (DPPH) discoloration assay described by Brand-Williams et al. (1995) with some modifications. The results of the TAA are reported as Trolox equivalents (TE) per 100 gram of fresh fruit weight.

Significant differences between treatments and the effect of retail and cool-store temperature were tested by a two-way analysis of variance. In figures and tables, the mean values followed by the same letter are not significantly different at $P \leq 0.05$.

RESULTS AND DISCUSSION

Gas composition in the MA packages

The O_2 content in both passively modified packages decreased rapidly during the first 24 hours, reaching 15.7% in the Xtend® film and 14.9% in the LDPE film (Fig. 1A). During the next two days, the decrease was very little and the final content of O2 in the Xtend® film was 15.1% and in the LDPE film 13.1%. In the actively modified LDPE package, the O_2 content increased slightly, being 12.4% after 24 hours and 11.9% at the end of cool storage.

The CO_2 content in the passively modified packages increased to 5.1% in the LDPE film and to 6.1% in the Xtend® film during the first 24 hours (Fig. 1B). The final CO_2 content in the LDPE film was 5.9% and in the Xtend® film 7.3%. In the actively modified LDPE package, the CO_2 content decreased rapidly, being 8.7% after 24 hours. At the end of cool storage, the CO_2 content in the active MAP was 5.9%, which was exactly the same as in the passive LDPE package.

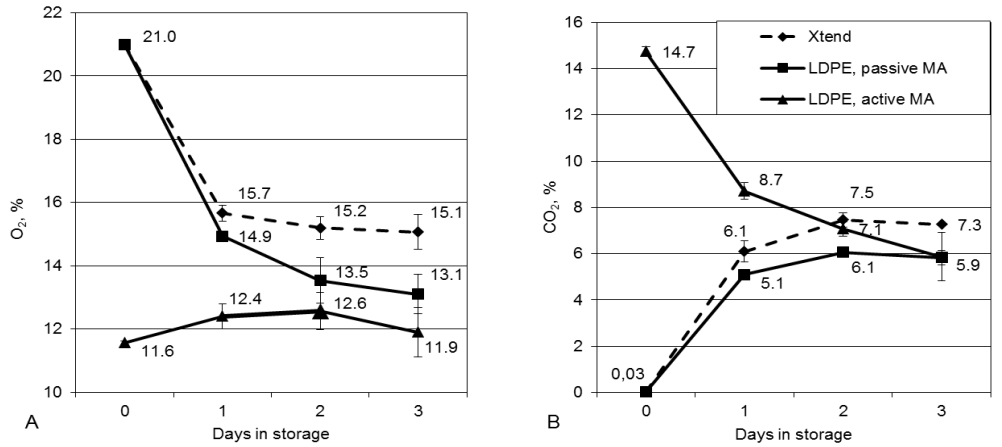

Figure 1. Changes in the O2 (A) and CO_2 (B) concentrations (% ± SD) in different modified atmosphere packages of 'Polka' raspberries stored at +1.6 °C for 3 days.

Fruit spoilage

The losses caused by rots were extensive in the present study, ranging from 9 to 23% (Table 1). The reason was obviously the extremely rainy weather during the fruit ripening period. The effect of the MA conditions on rotting in our trial was not significant. The mean effect of storage treatment showed that the Xtend® film decreased rotting compared to both LDPE films, but not compared to the control. Several authors have reported that high CO_2 concentrations (10, 20 and 30%) suppress rotting of raspberries (Agar & Streif, 1996; Haffner et al., 2002). In our trial, the CO_2 concentration in the passive MAP ranged from 5 to 7% and in the active MAP the CO_2 concentration also decreased below 10% after 24 hours. The mentioned CO_2 concentrations were probably too low to suppress rotting.

Table 1. The effect of different MA packages on fruit rotting, the SSC and TA of the raspberry 'Polka' after 4 days of storage in coolstore (+1.6 °C) and after 3 days in coolstore +24 hours in simulated retail conditions (+6ºC)

	Control	Xtend®	Passively modified LDPE	Actively modified LDPE	Mean
		Rotting weight, %			
Coolstore	16a	9a	16a	16a	14B
Retail	16a	13a	23a	22a	18A
Mean	*16AB*	*11B*	*20A*	*20A*	
		Soluble solids,%			
Coolstore	8.6b	9.3ab	9.7a	9.4ab	9.3A
Retail	9.2a	8.2b	8.7ab	9.1ab	8.8B
Mean	*8.9A*	*8.7A*	*9.2A*	*9.2A*	
		Titratable acids, %			
Coolstore	1.54b	1.66a	1.68a	1.53b	1.60A
Retail	1.50b	1.57a	1.48b	1.50b	1.51B
Mean	*1.52B*	*1.61A*	*1.58A*	*1.51B*	

Weight loss, dry matter and taste-related parameters

The average fruit DM content at harvest was 13.7 ± 0.4%. By the end of storage, it ranged from 12.7 to 13.3%. The effect of storage treatment on the DM content was not significant.

The weight loss of the control treatment ranged from 1 to 2% and in the MA packages from 0.1 to 0.2%. Thus, weight loss was not a limiting factor for berry quality during the experiment, since the loss according to Kenny (1975) needs to be at least 8% to cause a quality reduction. Haffner et al. (2002) also found in a trial with 5 raspberry cultivars that postharvest treatments did not affect fruit weight loss and dry matter content.

The initial SSC of the raspberries was 10.8 ± 0.4%, which is in agreement with the previously reported values for 'Polka' of 10.7–11.3% (Grajkowski & Ochmian, 2007). After storage, the SSC of the raspberries ranged from 8.6 to 9.7% (Table 1). In cool storage, the raspberries from the passive LDPE had retained the highest value of soluble solids. In retail conditions, the SSC was the highest in control fruit and the lowest in the Xtend® package. The average effect of storage temperature showed that 24 hours in retail conditions had decreased the SSC significantly.

At harvest, the raspberry TA was 1.84 ± 0.03% and after storage it ranged from 1.48 to 1.68% (Table 1). Grajkowski & Ochmian (2007) have reported the raspberry 'Polka' mean TA to be 1.75%, which is similar to our findings. After storage in cool conditions, the fruit TA was significantly higher in the passively modified LDPE and the Xtend® packages. In retail conditions, the TA content was higher in the Xtend® film. 24 hours in retail conditions had decreased the TA significantly.

Relatively high sugars and acids are required for good flavour of the fruit. The passively modified LDPE film maintained the highest SSC and TA in cool conditions, but not in retail conditions.

Bioactive compounds and total antioxidant activity

The AAC of the raspberry fruit before storage averaged 36 ± 3 mg 100 g FW^{-1}. Haffner et al. (2002) have studied the L-ascorbic acid content of 5 raspberry cultivars in Norway and the mean for all cultivars at the time of harvest was 23.2 mg in 100 g of berries. These results confirm that 'Polka' is a valuable cultivar with high ascorbic acid content. After 4 days of cool storage, the raspberry AAC had decreased and ranged from 22 to 27 mg 100 g FW^{-1} (Fig. 2). The AAC in the Xtend® film was lower compared to the LDPE films and the control after cool storage. As an average of the experiment, 24 hours in retail conditions did not cause a significant decrease in the AAC and no significant differences in the AAC between packages were found in retail conditions.

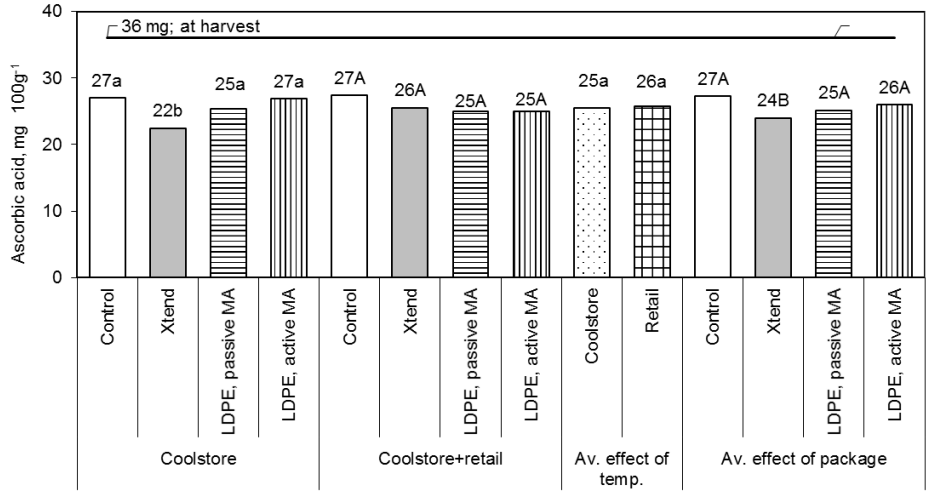

Figure 2. The effect of different MA packages on the ascorbic acid content of the raspberry 'Polka' after 4 days of storage in coolstore (+1.6°C) and after 3 days in coolstore +24 hours in simulated retail conditions (+6ºC).

The initial ACY content in the raspberries was 40 ± 3 mg 100 g FW^{-1}. Haffner et al. (2002) have found that the mean ACY level of 5 raspberry cultivars in Norway was 40 mg 100 g^{-1} fresh weight. After 4 days of cool storage, the fruit ACY content ranged from 34 to 43 mg 100 g FW^{-1} (Fig. 3). After cool storage, the raspberries held in the actively and passively modified LDPE packages had significantly lower ACY contents

compared to the control. Thus, in the MA packages, the ACY synthesis was slowed down. Haffner et al. (2002) also found that compared to the raspberries stored in normal atmosphere, the ACY content of the raspberries from controlled atmosphere conditions was significantly lower. Among other phenolic compounds, anthocyanins are derived from the phenylpropanoid pathway, which is activated by the enzyme phenylalanine ammonia-lyase (PAL). It has been demonstrated with table grapes (Romero et al., 2008) that CO_2-treated fruit have lower PAL activity and consequently also lower ACY content.

In retail conditions, the ACY content in both LDPE films had increased to the similar level with the control and the fruit from the Xtend® film had the lowest ACY content. The average effect of the last diurnal temperature was significant: as expected, warmer temperature increased the ACY content, which was not a favourable result in the particular cultivar. Darkening of the attractive red colour is considered to be one of the characteristics of quality loss in raspberries (Haffner et al., 2002). The fruit of the raspberry 'Polka' are of dark colour compared to some other cultivars. According to the experience of the growers, when selling 'Glen Ample' and 'Polka' fruit side by side, consumers prefer 'Glen Ample', which is of lighter colour. The dark colour of 'Polka' fruit is associated with overripeness. There are very few published studies identifying the sensory properties important to the consumer acceptance of raspberries. Villamor et al. (2013) have found that high colour intensity and green aroma were associated with negative drivers of liking of fresh raspberries.

Haffner et al. (2002) have stated that cultivars with a lighter red colour with less blue will keep a better colour after harvest compared to darker and more bluish cultivars.

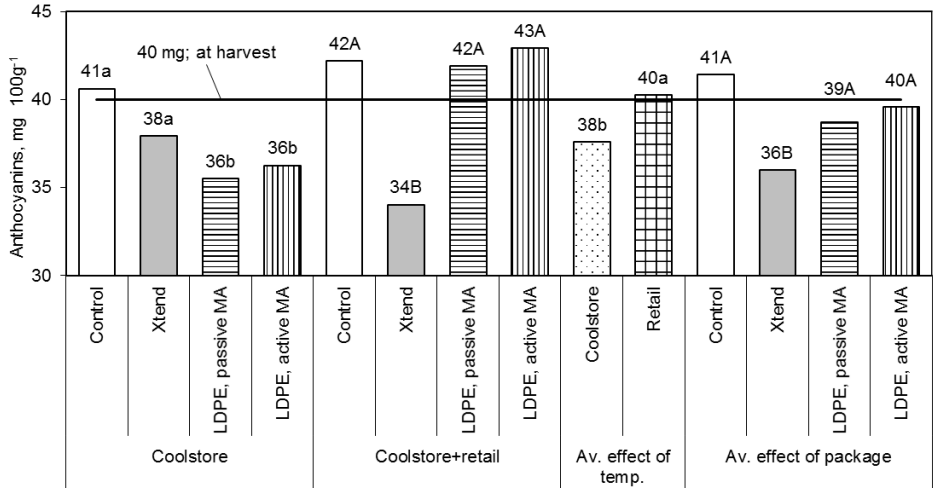

Figure 3. The effect of different MA packages on the anthocyanin content of the raspberry 'Polka' after 4 days of storage in coolstore (+1.6°C) and after 3 days in coolstore +24 hours in simulated retail conditions (+6ºC).

The TAA of the raspberries ranged from 95 to 154 TE mg 100 g FW^{-1} (Fig. 4). In cool conditions, the raspberries from the passively modified LDPE had the highest TAA, followed by the actively modified LDPE bag, the Xtend® bag and finally the

control, where the raspberry TAA was 32% lower than in the first mentioned treatment. In retail conditions, the situation had changed and the highest TAA was determined in the raspberries stored in the Xtend® package. Both LDPE bags had decreased raspberry TAA. The average effect of storage temperature was significant: 24 hours in retail conditions had significantly decreased the TAA. It has been reported that cyanidin-based anthocyanins with a *o*- dihydroxy moiety have the greatest antioxidant activity among anthocyanins (Rice-Evans et al., 1996). Therefore it was expected that in treatments where the ACY concentration was high, the TAA would also be high. For an unknown reason, negative correlation between the ACY and TAA was found. Schotsmans et al. (2007) carried out a controlled atmosphere storage experiment with rabbiteye blueberries and found that the antioxidant activity and total phenolic content were positively correlated when the berries were stored in a regular atmosphere, but negatively correlated after storage in a controlled atmosphere. The mechanisms for reversed correlation remain unclear.

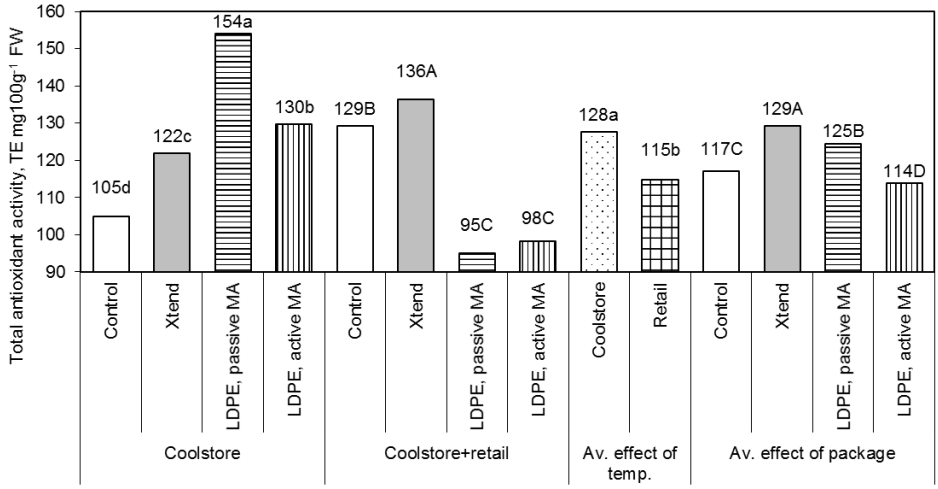

Figure 4. The effect of different MA packages on the total antioxidant activity of the raspberry 'Polka' after 4 days of storage in coolstore (+1.6 °C) and after 3 days in coolstore +24 hours in simulated retail conditions (+6ºC).

CONCLUSIONS

The modified atmosphere packages used in the current experiment did not suppress *Botrytis* rot probably due to the insufficient content of carbon dioxide (less than 10%). It would be worth trying other materials less permeable to oxygen and carbon dioxide in order to increase the CO_2 content to a sufficient level for suppressing microbial activity. However, the effect of MA on the fruit taste-related parameters and the antioxidant properties indicates the possibility to improve the consumer acceptability of 'Polka' raspberries by using modified atmosphere. The raspberries stored at 1.6°C, had the best quality in passively modified LDPE bags, since the fruit had higher SSC and TA and lower ACY content compared to the control, meaning that these fruits might have a more intensive taste and lighter red colour compared to others. After simulated retail conditions, the Xtend® bags turned out to be the most

suitable, since the fruit had the lowest ACY content (the fruit did not become too dark), but the highest antioxidant activity.

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REFERENCES

Agar, I.T. & Streif, J. 1996. Effect of high CO_2 and controlled atmosphere (CA) storage on the fruit quality of raspberries. *Gartenbauwissenschaft* **61**, 261–267.

Kenny, A. 1975. Handling strawberries and raspberries for fresh market II. Precooling. *Farm and Food Res.* **6**(3), 64–66.

Danek, J. 2002. 'Polka' and 'Pokusa' – new primocane fruiting raspberry cultivars from Poland. *Acta Hort.* **585**, 197–198.

Boyles, M.J. & Wrolstad, R.E. 1993. Anthocyanin composition of red raspberry juice: influences of cultivar, processing, and environmental factors. *J. Food Sci.* **58**, 1135–1141.

Brand-Williams**,** W., Cuvelier, M.E. & Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. *Food Science and Technol.* **28**, 25–30.

Cheng, G.W. & Breen, P.J. 1991. Activity of phenylalanine ammonialyase (PAL) and concentrations of anthocyanins and phenolics in developing strawberry fruit. *J. Am. Soc. Hortic. Sci.* **116**, 865–868.

Grajkowski, J. & Ochmian, I. 2007. Influence of three biostimulants on yielding and fruit quality of three primocane raspberry cultivars. *Acta Scientarium Polonorum Hortorum Cultus* **6**(2), 29–36.

Haffner, K., Rosenfeld, H.J., Skrede, G. & Wang, L. 2002. Quality of red raspberry *Rubus idaeus* L. cultivars after storage in controlled and normal atmospheres. *Postharvest Biol. & Technol.* **24**, 279–289.

Rice-Evans, C.A., Miller, N.J. & Paganga, G. 1996. Structure- antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* **20**, 933

Romero, I., Sanchez-Ballesta, M.T., Maldonado, R., Escribano, M.I. & Merodio, C. 2008. Anthocyanin, antioxidant activity and stress-induced gene expression in high CO_2-treated table grapes stored at low temperature. *J. Plant Phys.* **165**, 522—530.

Schotsmans, W., Molan, A. & MacKay, B. 2007. Controlled atmosphere storage of rabbiteye blueberries enhances postharvest quality aspects. *Postharvest Biol. & Technol.* **44**, 277–285.

Vetek, G. & Penzes, B. 2008. The possibilities of organic raspberry production – setting a Hungarian example. In Dimza, I. et al. (eds.). *Proceedings of the International Scientific Conference, Sustainable Fruit Growing: from Plant to Product*; 2008 May 28–31; Jurmala – Dobele, Latvia. Latvia State Institute of Fruit-Growing, pp. 233–242.

Villamor, R.R., Daniels, C.H., Moore, P.P. & Ross, C.F. 2013. Preference mapping of frozen and fresh raspberries. *J. Food Sci.* **78**, 911–919.

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Investigation of Casein Micelle Particle Size Distribution in Raw Milk of Estonian Holstein Dairy Cows

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Abstract. The particle size of milk influences its microstructure and defines many properties of dairy products such as colloidal stability, texture etc. Differences in particle size can significantly affect milk processing especially when membrane technology is used.
Aim of this investigation was to estimate casein micelle size in the raw milk of Estonian Holstein dairy cows and its variability concerning individual animals.
Milk samples were collected during 12 months with the interval of 25–35 days. DLS analyses were performed using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK).
Average mode of casein micelle particles size in raw milk of 44 cows was 171.13 nm with the variation range 70.1 nm and its distribution resembled a normal one. Casein micelles size mode of individual cows varied in a wide range from 148.5 (with variation range 18.2) to 194.1 (with variation range 27.6) nm which may be caused by differences in physiological and health status, stage of lactation and other factors concerning milk production.

Key words: casein micelle, dynamic light scattering, particle size, particle size distribution.

INTRODUCTION

Particle size (PS) and particle size distribution (PSD) gives valuable information about colloidal systems, among others milk and dairy products also (Beliciu & Moraru, 2009). Commonly used techniques for the analysis of PS and PSD are dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), scanning electron microscopy (SEM), size exclusion chromatography (SEC), cell electrophoresis, analytical ultracentrifugation (AUC) etc. Various analytical methodologies may give different results (Anema et al., 2005; Thu Tran Le et al., 2008; Dejan, 2010; Raza et al., 2011). Of the mentioned techniques, DLS is the most user-friendly and it gives relatively accurate and consistent results of protein samples which can be obtained in short period of time (Vasco et al., 2010). Main challenges to estimate casein micelle (CM) PSD by DLS, is the fact that measurements need to be performed with considerably pure solution and at low concentration of sample (Alexander & Dalgleish, 2006). For reliable estimation of CM PSD, 10 to 1 μg milk must be diluted in one millilitre of solution (Beliciu & Moraru, 2009). Casein micelle consists of four different protein fractions (α_{S1}-, α_{S2}-, β-, κ-casein). Each CM is covered with a layer of water molecules which affects the hydrodynamic diameter of micelle.

In former studies CM PSD had been mainly estimated in raw bulk milk, skimmed milk, reconstituted skimmed milk, lactose-free milk and pasteurized milk (Martin et al., 2007; Tran Le et al., 2008; Liu et al., 2013).

There is few published research which deal with CM in raw milk of individual cows, for example Bijl et al. (2014) investigated how milk chemical composition influenced casein micelle size of individual cows and de Kruif & Huppertz (2012) investigated how lactation stage affects CM PSD. The aim of current study was to approve suitability of dynamic light scattering (DLS) method for estimation of casein micelle PSD in raw milk and investigation of its variability of individual Estonian Holstein dairy cows during one year period.

MATERIALS AND METHODS

Raw milk samples of 44 Estonian Holstein dairy cows were collected at the Experimental Farm of Estonian University of Life Sciences (EMÜ) from January 2013 to December 2013, with the interval of 25 to 35 days. After milking samples were cooled down and stored at 5^{o}C, all analyses where made in the same day.

For stable results, samples were diluted at refrigerator temperature (5°C) just before the measurement using RPMI 1640 (PAA Laboratories GmbH, Pasching, Austria) as diluting media to concentration 1µg/ml, and filtered before measurement using a 0.45 µm, Ø 15mm Premium Syringe Filters (Agilent Technologies, Santa Clara, California). This procedure was necessary to remove larger particles such as fat, dust etc. 1500 µl of each sample was inserted into a single-use disposable sizing cuvette DTS0012 (Sarstedt REF 67.754, Sarstedt AG&Co, Nümbrecht, Germany). DLS analyses were performed using a Zetasizer Nano ZS analyzer (Fig. 1). The particle size estimations were made at fixed 173° backscattered angle using the default 'protein analysis mode' with automatic duration and four consequent measurements from a sample without delay. Automatic attenuation selection was switched off and number six was inserted as value for attenuator.

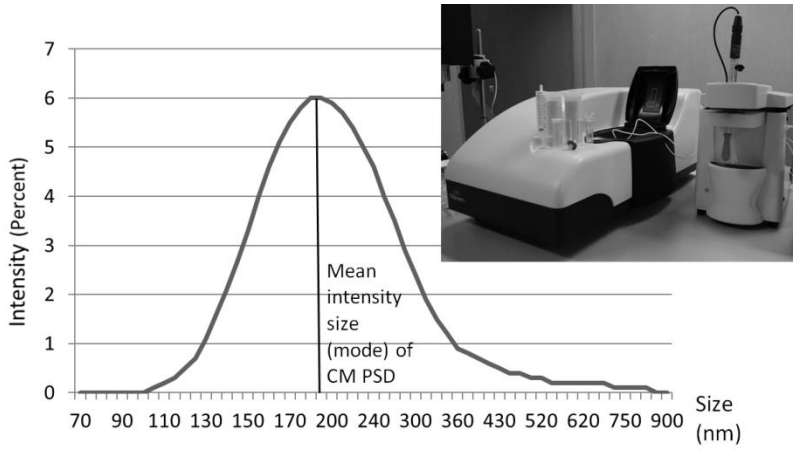

Figure 1. Typical example of CM PSD and Malvern Zetasizer Nano ZS analyser.

Measurement temperature was chosen 20°C, because this is a temperature commonly used in DLS measurements studies (Beliciu & Moraru, 2009). Data collection and first elaboration of these was carried out by Zetasizer software 7.01. The mode of PSD (mean intensity size of hydrodynamic diameter), average of total PSD (harmonic intensity of averaged particle diameter or Z-Average diameter) and all other data were exported to Microsoft Excel for further analyses. Typical example of CM PSD and measurement equipment are presented in Fig. 2.

RESULTS AND DISCUSSION

From obtained data only mean intensity of different variables of size was used. This index corresponds to the mode of CM PSD curve and represents the most essential information in it. Average of CM PSD modes of 328 samples was 171.13 nm with variation range 70.1 nm and it had standard deviation (SD) of 14.06. Histogram of these modes resembles normal distribution and covers the range 135–210 nm (Fig. 2).

More than half of the modes (58.5%) covered the range 155–175 nm. Tails of this modes distribution were represented by four (1.2%) samples in range from 135–140 nm and six (1.5%) samples in the range 200–210 nm. Former studies about CM PSD in bulk (and treated) milk by different methods showed up quite the same variability in average mode of CM PSD: 150–200 nm (Table 1). Some variation in results can be explained by different measurement methodologies used for preparation of probes and by differences in milk itself (bulk milk of different production system, cows breed etc.).

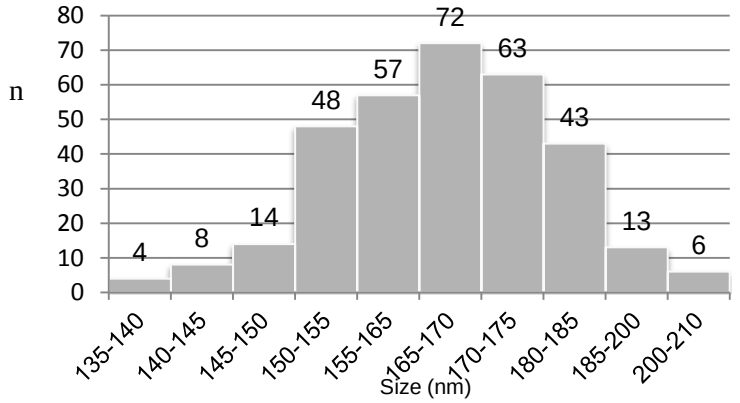

Figure 2. Histogram of CM PSD modes in raw milk probes of Estonian Holstein dairy cows.

Studies of Beliciu & Moraru (2009) and Liu et al. (2010) are in agreement with results of our investigation. They found that CM PSD average in treated bulk skim milk varies from 176.3 to 178.8 nm. This result is only somewhat bigger than average mode of CM PSD in our study. Overall accordance of above mentioned studies with our investigation suggests that DLS method is suitable for estimation of CM PSD in raw milk of cows too.

To investigate the role of individual animals on mode of CM PSD, all data were rearranged into groups by cow number. Of all animals under this study (n = 39) milk of

six cows has been analysed 3–5 times and milk of 33 cows 6–11 times during the total investigation period.

Table 1. Summary of former studies about the CM size in milk

Author	Method	Mode of CM size, nm
Tran et al., 2008	NTA	192
Martin et al., 2007	Cell electrophoresis	185
Tran et al., 2008	DLS	186
Liu et al., 2010	DLS at 20°C	177
	DLS at 40°C	200
Beliciu and Moraru, 2009	DLS at 20°C	176
	DLS at 50°C	194
De Kruif and Huppertz, 2012	DLS	154–230
Raza Hussain et al., 2011	DLS (solvent NaCl)	150

Differences in number of analyses were caused by changes in lactation stage mainly.Individual dairy cows' average mode of CM PSD in descending order is presented in Fig. 3 and numerical values of modes and their variations are given in Table 2.

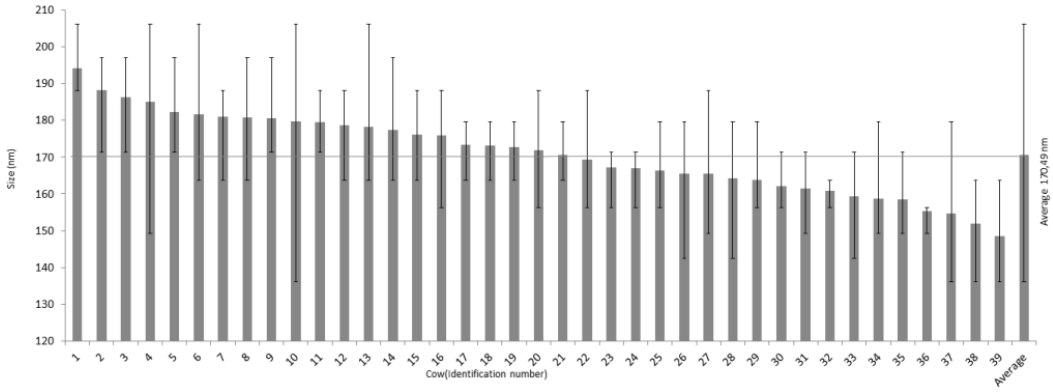

Figure 3. Individual dairy cows average mode of CM PSD in descending order.

Average mode of CM PSD in this study varied in range from 148.5 nm to 194.06 nm (group average of 39 cows was 170.95 nm, with variation range of 27.40 nm and SD of 19.4). This data is similar to the results obtained in analyses about the total herd (44 cows/328 samples).

Largest average mode of CM PSD in this dataset was 194.06 nm, with 9.1 nm variation and the smallest one had PSD of 148.5 nm with 27.6 nm variation. In Fig. 4 casein micelle PSD variability of five individual cows are presented. Cow No. 1 and cow No. 39 average mode of CM PSD have extreme values (min and max), three other cows (10, 18, 32) have average mode closer to mean of the herd. Average mode value of CM PSD and its variation of different cows seem to be independent from each other. It was confirmed by statistical analyses – they showed up only a very slight positive correlation (0.195). Also increasing number of samples does not make this relation

better. For example, by 10 cows of 11 which had 10 CM PSD estimations each, variation mean value (19.4 nm) of the mode was exceeded. The only reasonable explanation to that may be found in cows' individuality.

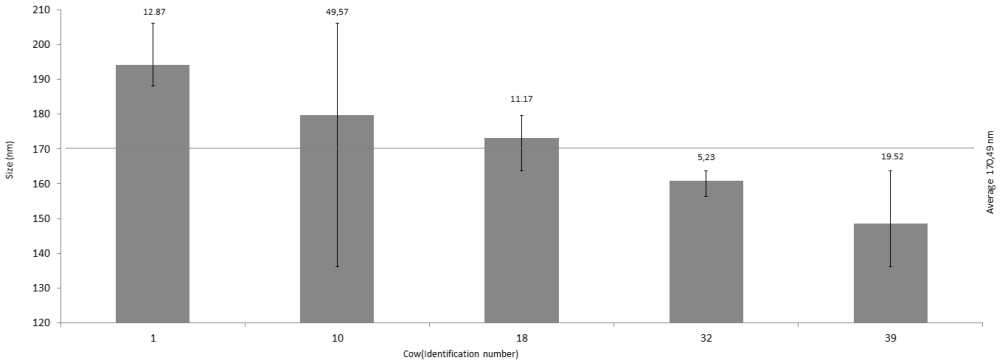

Figure 4. Casein micelle PSD variability of individual cows 1, 10, 18, 32 and 39.

Table 2. Average modes of CM PSD in milk of individual cows in descending order, their variations and standard deviations

Cow identification number	Mode of CM PSD	Variation range of CM PSD	SD of CM PSD	Count of samples	Cow identification number	Mode of CM PSD	Variation range of CM PSD	SD of CM PSD	Count of samples
1	194.1	18.2	12.9	3	**21**	170.6	15.8	11.2	9
2	188.3	25.5	18.0	6	**22**	169.3	31.7	22.4	10
3	186.2	25.5	18.0	9	**23**	167.2	15.1	10.7	9
4	184.9	57.0	40.3	9	**24**	167.0	15.1	10.7	7
5	182.2	25.5	18.0	10	**25**	166.4	23.2	16.4	9
6	181.7	42.5	30.1	10	**26**	165.6	37.0	26.2	10
7	180.9	24.3	17.2	7	**27**	165.4	38.8	27.4	11
8	180.8	33.2	23.5	9	**28**	164.3	37.0	26.2	7
9	180.5	25.5	18.0	10	**29**	163.8	23.2	16.4	9
10	179.7	70.1	49.6	9	**30**	162.1	15.1	10.7	9
11	179.6	16.6	11.7	6	**31**	161.4	22.2	15.7	3
12	178.7	24.3	17.2	9	**32**	160.7	7.4	5.2	5
13	178.2	42.5	30.1	10	**33**	159.5	28.9	20.4	10
14	177.3	33.2	23.5	10	**34**	158.7	30.3	21.4	10
15	176.0	24.3	17.2	9	**35**	158.6	22.2	15.7	10
16	175.8	31.7	22.4	8	**36**	155.4	7.1	5.0	8
17	173.2	15.8	11.2	9	**37**	154.6	43.4	30.7	10
18	173.1	15.8	11.2	5	**38**	152.0	27.6	19.5	3
19	172.7	15.8	11.2	7	**39**	148.5	27.6	195	7
20	171.9	31.7	22.4	3	**Average**	170.9	27.4	19.4	8.1

Also the absence of tight correlation between CM PSD average mode and its variation refers to influence of certain factors connected to cows' individuality (changes in physiological status, disease incidences, stages of lactation, etc). All these aspects should be topics of further investigations.

CONCLUSION

For the first time, studies of casein micelle PSD variability in milk samples of individual Estonian Holstein dairy cows have been carried out by DLS measurements during one year period. The main results of this study can be summarized as follows: 1) Average mean intensity (mode) of CM PSD in raw milk of Estonian Holstein dairy cows was 171.13 nm and its variation (range 135–210 nm) resembled statistically normal distribution. 2) Weak correlation between CM PSD average mode and its variation in milk samples of individual cows may refer to the possible influence of cows' physiological status, disease incidences and stages of lactation etc. which will be studied in further research.

REFERENCES

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

Anema, S.G., Lowe, E.K. & Stockmann, R. 2005. Particle size changes and casein solubilisation in high-pressure-treated skim milk. *Food Hydroc.* **19,** 257–267.

Beliciu, C.M. & Moraru, C.I. 2009. Effect of solvent and temperature on the size distribution of casein micelles measured by dynamic light scattering. *J. Dairy Sci.***92**, 1829–1839.

Bijl, E., de Vries, R., van Valenberg, H., Huppertz, T. & van Hooijdonk, T. 2014. Factors influencing casein micelle size in milk of individual cows: Genetic variants and glycosylation of k-casein. *Int. Dairy J.* **34**, 135–141.

Dejan, A. 2010. *Dynamic light scattering and application to proteins in solutions*. University of Ljubljana Faculty of Mathematics and Physics, Department of Physics, Ljubljana, 19 pp.

Kruif de C.G. (Kees) & Huppertz, T. 2012. Casein Micelles: Size Distribution in Milks from Individual Cows. *J. Agric. Food Chem.* **60**, 4649−4655.

Liu, D.Z., Weeks, M.G., Dunstan, D. E. & Martin, G.J.O. 2013. Temperature-dependent dynamics of bovine casein micelles in the range 10–40°C. *Food Chem.* **141**, 4081–4086.

Martin, G.J.O., Williams, R.P.W. & Dunstan, D.E. 2007. Comparison of Casein Micelles in Raw and Reconstituted Skim Milk. *J. Dairy Sci.* **90**, 4543–4551.

Raza, H., Claire, G. & Joël, S. 2011. Revealing Casein Micelle Dispersion under Various Ranges of Nacl: Evolution of articles Size and Structure. *Eng. Tech.* **51**, 972–982.

Tran, Le T., Saveyn, P., Hoa, H.D. & Van der Meeren, P. 2008. Determination of heat-induced effects on the particle size distribution of casein micelles by dynamic light scattering and nanoparticle tracking analysis. *Int. Dairy J.***18**, 1090–1096.

Vasco, F., Andrea, H. & Wim, J. 2010. Critical Evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the Measurement of Nanoparticles and Protein Aggregates. *Pharm. Res.* **27**(5), 796–810.

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Fermented sauces for child nutrition from age three

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Abstract. One of the directions in modern food science is the development of diets for children of different age groups based on their physiological needs and psycho-emotional activity. One of the approaches to solving this problem is functional product design. Such products are not medicaments, but help to prevent the diseases and ageing processes of the human organism.
The purpose of our research was to develop fermented sauce compositions based on milk and plant ingredients for child nutrition.
Mass fractions of pumpkin and banana purée, berry syrup and stabilizing additives were experimentally determined.

Key words: Fermented sauces, child nutrition, fruit and vegetable purées, berry syrups, corn starch.

INTRODUCTION

The creation and launching of functional food products is one of the directions of the human nutrition program initiated by the UN. Even in the developed countries there is a deficit in biologically active substances, macronutrients and micronutrients, and ballast substances in the diet. Functional food products which are not medications help to prevent diseases and ageing. 65% of the modern market of functional food products consists of dairy products (Zakharova & Mazeeva, 2010).

One of the priorities in the field of healthy eating is the existence of a varied range of products for functional purposes. Vegetable raw materials with a wide range of biologically active substances (vitamins, minerals, dietary fiber, antioxidants, etc.) are currently widely used. The presence of these ingredients improves many physiological processes in the body and improves the immune system. One of the most accessible ways to create products with such qualities is the development of technology for the production of functional foods with a complex mineral composition. The creation of multicomponent products is mainly aimed at the regulation of amino acids, lipids, carbohydrates, minerals and vitamins in human diet. (Tikhomirova, 2009). Children and elderly people have become an increasingly important segment of the consumers and such products are aimed specifically at these two groups (Murray et al., 2001).

The present study focuses on the development of safe functional foods intended for children. A balanced diet is one of the conditions for the normal development of resilience in children, helping to cope with exposure and infection. Baby food plays a significant role in ensuring children access to balanced products, the production of

which is possible only in the conditions of specialized modern enterprises. The most important are the products included in the daily diet. A special group of them is sauces. Due to the presence of extractive, aromatic and flavoring substances stimulating the secretion of digestive glands, sauces are a good source of key components in food (Tarasova & Taghiyev, 2009).

At present, various types of sauces and soups are extremely popular in the consumer market because of the significant expansion of the average consumer basket. However, not all products in this category, available now in the market, are ideal for the children. The abundance of these products is unacceptable for children because of their content of additives, stabilizers, preservatives, and artificial dyes. That is why the study is so important in the current situation in the food market, as regards children of pre-school and school age. According to the Institute of Nutrition of Russian Academy of Medical Sciences, a broad-scale monitoring of the nutritional status of school-age children has shown a shortage of high-grade proteins (30–70%), fat (10–40%), and vitamins (40–70%). The vast majority of pupils (about 80%) are eating at school cafeterias and buffets during school hours. Due to deficient school meals, according to the I.M. Sechenov Moscow Medical Academy, 37% of children regularly experience hunger and 39% from time to time. Because of considerable mental stress at school, the well-being of pupils depends on regular meals and a high-calorie diet. Childhood and adolescence is characterized by relatively high energy consumption. Thus, the energy consumption of children 7–10 years of age constitutes about 80 kcal kg^{-1} of body weight, 13–16 year old adolescents 50–65 kcal kg^{-1}, and adults 45 kcal kg^{-1}.

The recommended consumption rates of basic nutrients for children and adolescents by age and gender according to the Institute of Nutrition and the sanitary standard 2.4.5.2409-08 ‘Hygienic requirements for catering pupils in general educational institutions, institutions of primary and secondary professional education’ are shown in Table 1.

Table 1. Recommended consumption rates of basic nutrients for children and adolescents according to age

Poultry nutrition	Quantity, per day	
	7–11 years	11–18 years
Protein, % of calories	14	13
Fat, % of calories	30	30
NLC, % of calories	10	10
Sugar, % of calories	10	10
Calcium, mg	1,100	1,200
Vitamin C, mg	80	100
Energy value, kcal	2,400	2,800

Feeding children of school and pre-school age is an important task. In fact, it has become increasingly difficult to work out a balanced and safe diet for the children due to the abundance of the latest developments of food industry filling the store shelves. Not all of these innovations are suitable for children, but the children prefer such products because of their pleasant organoleptic properties. However, there is a way out. The new products can be both useful and valuable in terms of nutritional value, but at the same time tasty foods with health benefits that babies could drink every day. The

younger generation is fond of sweet dairy desserts. Therefore, one solution to the shortage of dairy products in the diet of children of pre-school and school age may be the development of new functional milk based products, which could easily be combined with their taste preferences. Sauce could be ideal as such a product, as it is both fashionable and popular.

MATERIALS AND METHODS

A study on the development of composition and technology of fermented sauces for three year old infants was conducted in the laboratory of technology of milk and food biotechnology at Institute of Refrigeration and Biotechnology of Saint Petersburg National Research University of Information Technologies, Mechanics and Optics.

The objects of research were:

- skimmed milk powder with a fat content of 1.25%;
- homogenized mashed pumpkin 'Umnitsa' for early childhood (Ivanovo plant of baby food);
- homogenized banana purée for early childhood (Ivanovo plant of baby food);
- blueberries, sugar free syrup 'Huckleberry' ('Petrodiet');
- viburnum berry syrup ('Vifiteh');
- corn starch premium sort ('Starch-center');
- sourdough of pure cultures of viscous *Streptococcus thermophilus*.

Milk powder was used in the experiment with the aim of eliminating differences in physical and chemical indicators of raw milk. However, at the implementation of fermented sauce into production, it is recommended to use raw milk as a base.

Raw materials for the manufacturing of fermented sauces must meet the microbiological requirements presented in Table 2. The milk used for manufacturing baby food products should not be below the first grade. Manufactured fillers comply with microbiological criteria, as do products for babies from 6–10 months.

Table 2. Acceptable levels of bacteria and somatic cells in raw milk

Product milk, grade	KMAFAnM	Weight of the product, which is not allowed		Max content of somatic cells
		Coliform bacteria	Pathogens, including *salmonella*	
Supreme	$1*10^5$	–	25	$4*10^5$
the first	$5*10^5$	–	25	$1*10^6$
the second	$4*10^6$	–	25	$1*10^6$

Integrated study design adopted these methodologies:

1. Determination of active acidity;
2. Determination of dry matter and mass fraction;
3. Definition of syneresis properties.

Determination of active acidity

Potentiometric method is based on the measuring of voltage between two electrodes (electrode measurement and comparison). Electrodes couple before the measurement and should be placed in a glass with a solution of hydrochloric acid molar concentration of 0.1 mol/dm^3 in intervals between measurements. Electrodes are washed by distilled water before tests and sediment particles must be removed through filter paper.

We poured a 40 ± 5 cm^3 sample of milk or liquid product in a glass with a capacity of 50 cm^3 at the temperature 20 ± 2°C and dipped in the lead pair. After that we took the readings.

The measurement of pH in each sample is repeated twice, each time removing the electrodes from the sample and immersing them into the product.

The immersion depth of the electrode pair into the glass during the trial shall be not be less than 30 mm and that of combined electrodes no less than 16 mm. It is not allowed to touch the electrodes or the glass of the temperature compensator bottom and sides.

After the measurement of each sample of milk or dairy products, an electrode couple should be rinsed with distilled water at the temperature 30–40°C and the sediment particles in the distilled water must be removed through filter paper.

The final results of the measurement of active acidity in milk and dairy products are obtained by taking the arithmetic mean of the results of two parallel determinations.

Determination of moisture and dry matter content in sauces

It is necessary to put a doubled cheesecloth in an aluminum weighing bottle, then to exsiccate the content with an open lid at 105°C for 20–30 min, after that to cool it in the desiccator for 20–30 min by closing the lid, and then to weigh the content.

3 ml of the investigated product should be taken into a prepared aluminum weighing bottle pipette, distributed at regular intervals over the entire surface of the doubled cheesecloth and weighed after closing the lid. Then open aluminum container and lid in the oven at 105°C for 60 min, after which the aluminum container is closed, cooled and weighed. Drying and weighing continue during 20–30 min until the difference in mass between two successive weighings is no more than 0.001 g.

Definition of syneresis properties

The study of syneresis properties of clots was conducted as follows: a 10 ml test sample was placed into a measuring tube and centrifuged for 30 min, noting every 5 min the precipitated serum volume. By results of the research the rate of sample syneresis was found out and the ability of serum clotting was concluded.

Research organization

In the course of this study, we carried out experiments on pumpkin and banana puree and syrup percentage, as well as on the doses of stabilizer for a product with high organoleptic properties and a texture typical of this type of products. The control sample of sauce No 1 was prepared on the basis of raw milk without the addition of plant fillers and was produced from banana purée; the control sample of sauce No 2 was prepared from pumpkin purée.

All samples of sauces were made on the basis of skimmed milk, reduced to a mass fraction of solids 16%, fermented together with pure cultures of lactic acid of viscous *Streptococcus thermophilus*. Before developing, all the samples were subjected to intensive mixing in a blade mixer at the speed of 800 RPM to prevent stratification of the foundations and filler. Further samples were put in a thermostat at the temperature 44 ± 2°C and fermented for 4–6 hours. The finished product was sent ripening to the holding room at 4 ± 2°C.

RESULTS AND DISCUSSION

The dynamics of decreasing the acidity of sauce samples during the process of fermentation was studied.

In the first stage of the research the effect of doses of applied plant fillers on the fermentation of samples was investigated. Fig. 1 shows the use of different doses of pumpkin purée added in the amount of 15, 30, 45% of the mass of the initial mixture.

Fig. 1 shows that the fermentation all samples lasted for approximately six hours. It should be noted that the change in acidity was faster in the control sample, as skimmed milk is a suitable environment for the development of starter microflora. The graph also shows the reduction in active acidity depending on the doses of filler: the higher the dose, the slower pH decreases. This may be due to the presence of large quantities of organic acids in samples with high filler content, which impede the rapid multiplication of the microflora of the leaven. However, adding a filler up to 30% of the amount had a slight effect on the fermentation of the mixture, which is why further investigation used a dose of about 30% for making a plant component.

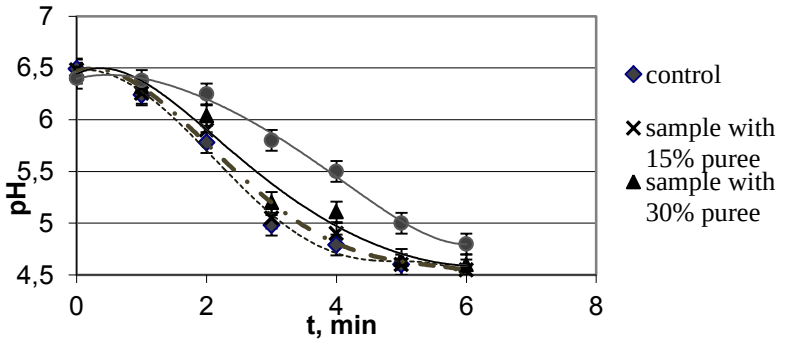

Figure 1. Change in the active acidity of samples with different doses of added pumpkin purée.

Banana and pumpkin purée were selected as plant components. Fig. 2 shows the variation in the active acidity of the selected samples in comparison with the control sauce.

The initial values of the active acidity of the samples with fillers are lower than in the control sample. But the development of the starter microflora occurs rapidly in the control sample as noted previously. However, after 6 hours of fermentation, samples reach similar values of active acidity. This allows establishing the necessary time for the fermentation of all samples, which is 6 hours.

The study should also figure out how to affect the process of fermentation by adding viburnum syrup as a sweet component of the recipe. Fig. 3 shows the effect of

syrup (in an amount of 9%) on the fermentation process of the sample with pumpkin purée.

Fig. 3 shows that the decrease in active acidity is faster in the sample without syrup. It is obvious that a high content of sugar and organic acid does not make the microflora of the starter culture evolve quickly and seamlessly in the sample with syrup, and as a result, the fermentation occurs at a slower speed compared to the sample without syrup. It should be noted that samples obtained in both the first and second experiments had a good flavour and taste but the sauces lacked the required consistency. At the end of the fermentation the samples had the sleek, glossy clot, but a heterogeneous structure: when mixing, there was a small separation of the serum that affected the consistency of the product.

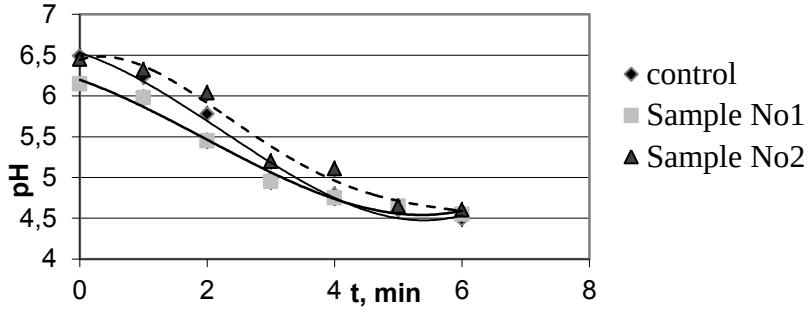

Figure 2. Change in the active acidity of samples with different fillers.

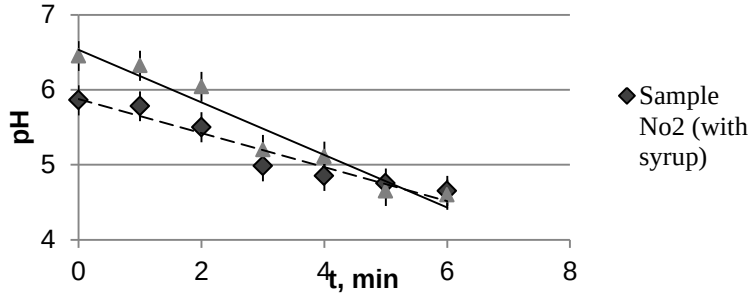

Figure 3. Change in the active acidity of samples with addition of syrup and without syrup.

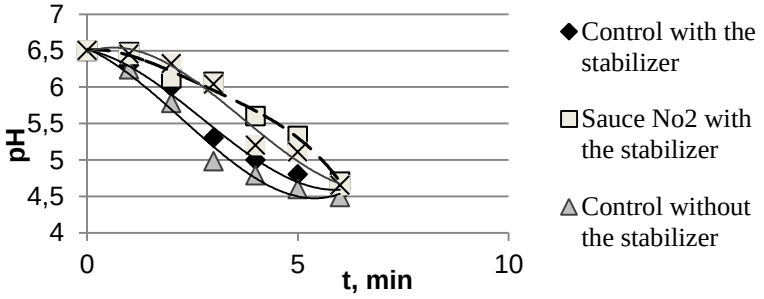

Figure 4. Change in active acidity of samples with and without stabilizer.

Consequently, it appears to be necessary to add a stabilizer in the manufacturing process of the fermented sauces. Fig. 4 presents diagrams of decrease in active acidity during fermentation of samples (control sauce and sample No2) with different fillers with and without a stabilizer. The stabilizer was added according to the recommendations in the amount of 2.7%.

On comparing these graphs we can say that the presence of the stabilizer does not affect the fermentation of the samples significantly. However, with the introduction of the stabilizer, we are reaching the desired consistency and structure of samples. Thus, the inclusion of a stabilizer in the formulation of sauces is appropriate.

Study on syneresis properties of samples.

Syneresis properties are an important quality indicator in the production of fermented products. It is required that the allocation of serum during centrifugation be minimal (V, %).

As a result of this experiment, the following data presented in Fig. 5 were obtained.

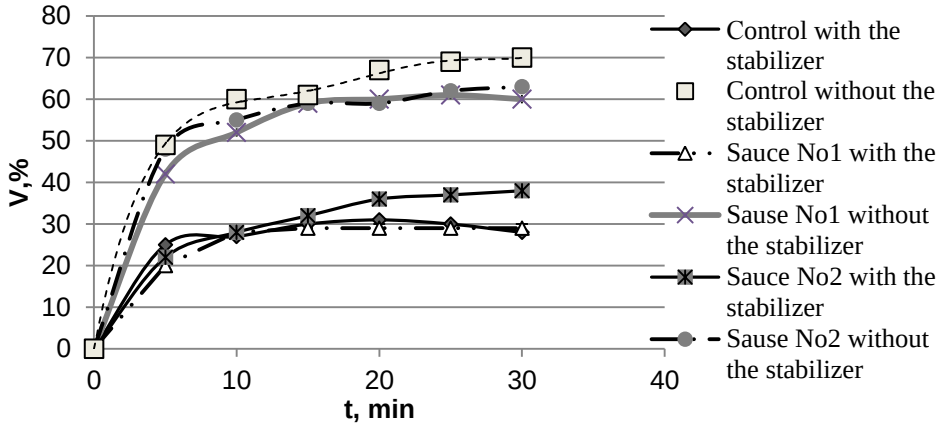

Figure 5. Syneresis properties of sauce samples with and without stabilizer.

Fig. 5 shows the syneresis properties of test samples with and without stabilizer. The graph shows the apparent stabilizing effect of structure with stabilizer: samples with the stabilizer separated the serum significantly less than samples without the stabilizer.

Samples No1 and 2 without the stabilizer are more resilient than the control sauce without the stabilizer. This may be due to the stabilizing quality of dietary fiber, which is part of banana and pumpkin purée.

Composition and properties of developed sauces

While working out the sour sauce recipe, it was decided to use 34–37% of plant component ingredients of the total weight of the finished product. Such a filler dose provides the necessary organoleptic qualities and functionality without violating the regulations for child nutrition products.

In order to obtain the desired consistency of the product of this type and the stabilization of the system, it was decided to introduce corn starch in the amount of 2.7% of the total weight of the sauce as a stabilizer.

Recipes for cooking sauces No 1 and No 2 for 1,000 kg of product are presented in Tables 3, 4.

Table 3. Recipe of sour sauce No 1 with banana purée for 1,000 kg of product (excluding losses)

Component	Weight, kg
Water	515.0
Banana purée	283.0
Skimmed milk powder	69.0
Blueberry syrup	61.0
Sourdough (viscous thermopile *Streptococcus*)	45.0
Stabilizer (corn starch)	27.0

Table 4. Recipe of sour sauce No 2 with pumpkin purée for 1,000 kg of product (excluding losses)

Component	Weight, kg
Water	496.0
Pumpkin purée	273.0
Skimmed milk powder	68.0
Viburnum syrup	91.0
Sourdough (viscous thermopile *Streptococcus*)	45.0
Stabilizer (corn starch)	27.0

The moisture content of the dry matter is an important characteristic of the finished product. The data obtained are summarized in Table 5.

Table 5. Moisture content of dry matter in sauce samples

Samples	Dry matter content, %	Moisture content, %
Control sample	14.9 ± 0.2	85.1 ± 0.2
Sauce No 1	17.1 ± 0.2	82.9 ± 0.2
Sauce No 2	14.2 ± 0.2	85.8 ± 0.2

The present study also examined the organoleptic parameters of the samples.

Children have been documented to have different taste thresholds than adults (Glanville et al., 1964; Hermel et al., 1970). It is apparent that children have different dietary habits and preferences than adults. Whether this is a result of differences in perception *per se*, familiarity, a learned behaviour or a combination of these, is not yet clear. It may therefore be desirable to train children for descriptive profiling or investigate these implications more thoroughly (Moskowitz, 1994; Chen & Resurreccion, 1996; Baxter et al., 1998).

As it was not possible to teach children sensory analysis in the present study, organoleptic evaluation of the received sauce samples was carried out by a group of 8 people from the academic staff working at the Department of Milk Technology and Food Biotechnology. The panelists were trained in accordance with the ISO 8586-1: 1993.

Preparation and use of samples for sensory analysis was carried out according to the recommendations (Seon-Suk et al., 2012). The evaluation was conducted for each indicator on a five-point system. Samples were served with a glass of water after they were left at the room temperature for 10 min. Samples were presented at the same time in each session. Sensory evaluation of samples in each analysis was carried out 2 times. The control points of organoleptic evaluation were taste, colour, and consistency. Results of the evaluation are summarized in Tables 6 and 7 and displayed on the chart.

Table 6. Results of organoleptic evaluation of fermented sauces with different fillers

Samples	Points			
	Sour milk taste	Taste filler	Colour	Consistency
Sample No 1	4.25	4.88	4.13	4.75
Sample No 2	4.38	4.88	4.88	4.88

Sample No 2 got the highest score in organoleptic evaluation of the best consumer properties; however, sample No 1 received high praise too, particularly for its sour milk taste, taste filler and consistency, losing only in colour. The latter is not substantial enough to discard the sample from further research. The samples must meet the requirements shown in Table 7.

Table 7. Organoleptic characteristics of fermented sauces with different fillers

Organoleptic characteristics	Sauce with mashed banana and blueberry syrup	Sauce with pumpkin purée and cranberry syrup
Taste and flavor	Sour milk, sweet, with a strong banana taste and flavour of blueberries	Sour milk, sweet, with a pronounced taste of pumpkin and cranberry flavour
Consistency	Homogeneous, viscous	Homogeneous, viscous
Appearance	Homogenous mass	Homogenous mass
Colour	Beigish-grey	Bright orange

Table 8. Microbiological safety indicators for sauces

The microorganisms identified	Sauces for children's and dietary nutrition
Spore *mesophilic* aerobic and facultative anaerobic-team *B.subtilis*.	Meet the requirements of the industrial sterility. In the case of determining the number of these micro-organisms, it should be no more than 11 cells in 1 g (cm^3) of the product
Mesophilic Clostridium	Do not meet the requirements of the industrial sterility detected in 10 g product (cm^3)

Fermented sauces must meet the requirements ‘Hygienic requirements for safety and nutritional value of foods. Public health regulations 2.3.2.1078-01’ shown in Table 8.

CONCLUSION

The study was carried out to develop the composition and technology of fermented sauces for children from age three. Based on the results of the research we can make the following conclusions:

1) The possibility of using filling materials of plant origin for the production of fermented sauces was proved: it is possible to use banana and pumpkin purée and berry syrup for this purpose in an amount of 34–37% of the total weight of the finished product. It also provides a perspective for enlarging the assortment of fermented sauces by introducing other combinations of fruit or vegetable purées and berry syrups;

2) The viscous thermopilus culture of Streptococcus (*Streptococcus thermophilus*) promotes the clot formation of moderately viscous consistency with a pleasant sweet taste of fermented sauces;

3) The use of corn starch as stabilizer allows to achieve the desired consistency and structure of sauces: samples with added starch separated almost 2 times less whey than samples without stabilizers;

4) The requirements for fermented sauces with various fillings were identified. The organoleptic characteristics: taste of fermented milk with a pronounced taste of fillers, aroma with a pronounced flavour of fillers; consistency is homogeneous, viscous.

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REFERENCES

Baxter, I.A. & Jack, F.R. & Schro der, M.J.A. 1998. The use of the repertory grid method to elicit perceptual data from primary school children. *Food Qual. Prefer*. **9**(2), 73–80.

Chen, A.W. & Resurreccion, A.V. (1996). Age appropriate hedonic scales to measure food preferences of young children. *J. Sens. Stud*. **11**, 141–163.

Dobrynina, E.S. & Lomovskij, O.I. 2010. Development of new formulations of sauces and dressings functional purpose. *Food industry*. 54–55.

ISO 8586-1: 1993 (Sensory analysis. General guidance for the selection, training and monitoring of assessors, Part 1. Selected assessors.

Glanville, E.V., Kaplan, A.R. & Fischer, R. 1964. Age, sex and taste sensitivity. *J. Gerontol*. **34**, 834–840.

Hermel, J., Schonwetter, S.V. & Samueloff, S. 1970. Taste sensation and age in man. *Journal of Oral Medicine*. **25**, 39–42.

Moskowitz, H.R. 1994. Children versus adults. Food concepts & products. *CT: Food & Nutrition Press*. 293–331.

Murray, J.M., Delahunty, C.M. & Baxter, I.A. 2001. Descriptive sensory analysis: past, present and future. *Food Res. Int*. **34**: 461–471.

Seon-Suk J., Seung-Joo L., Palanivel G. & Hae-Soo K. 2012. Comparative Study of Flavor, Texture, and Sensory in Cream Cheese and Cholesterol-removed Cream Cheese. *Food Sci. Biotechnol*. **21**(1), 159–165.

Tarasova, L.I. & Taghiyev, T.G. 2009. Sauces and mayonnaises-is there a difference. *Oil and fat industry*. 7. (In Russian).

Tikhomirova, N.A. 2009. Current state and prospects of development of functional food products. *The dairy industry*, 5–6. (In Russian).

Tikhomirova, N.A. 2009. Healthy eating for students. *The dairy industry*.78–79. (In Russian).

Tikhomirova, N.A. & Churakov, M.M. 2007. Functional fermented sauces. *The dairy industry*. **3**, 59–60. (In Russian).

Zakharova, L.M. & Mazeeva, I.A. 2010. Technology of new functional foods milk based. *Storage and processing of agricultural raw materials*. 63. (In Russian).

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The unique characteristics of milky-wax ripe walnuts and their usage

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Abstract. The objective of the research is to obtain biologically valuable and safe food products, which have functional qualities and extended shelf life by using the antimicrobial properties of walnuts and walnut leaves of milky-wax ripeness. A study was conducted on the possibility of using walnuts of milky-wax ripeness (walnut materials) in the production technology of functional foodstuffs (cheese products, beverages, cheese, desserts, and bakery products). The study identified types and possible amounts of walnut additives into the developed products. Various additives were obtained during the research, such as extracts, tinctures, dry powders, and capsules. The most unique specific substance in raw nut is juglone. Juglone is a natural antibiotic that inhibits more than 100 kinds of pathogenic microorganisms. The developed products with the addition of walnuts and walnut leaves of milky-wax ripeness contain high amounts of vitamins (especially C, E, A, and group B) and minerals (iodine and manganese). For this reason, walnut contains the natural antibiotic juglone, which increases the shelf life of products up to 21 days without the use of stabilizers or preservatives. Because of the multifunctional properties of raw nut, more specifically the antibacterial, fungicidal and preservative properties of juglone, the additives ensure a sustainable microbiological state of the products. Juglone allows sparing technological heat treatment of raw milk (40 ± 2°C) that reduces energy costs for the operation and the equipment. Various population groups can successfully use the designed products as functional food; what is more, they can be used for medical purposes.

Key words: Walnut, fermented products, dairy products, juglone, preservative, milky-wax ripeness.

INTRODUCTION

Walnut is a unique vegetable raw material, all parts of which can be used by humans. Already the ancient Greeks and Romans mentioned walnut. Theophrastus, 'the father of botany', was one of the first to describe walnut. This plant is mentioned in the writings of Cicero, Pliny, Virgil, and Hippocrates (Derzhavina, 2000). Michuryn called walnut a tree, which is like an industrial complex, as absolutely all its parts are used by humans: ripe and unripe fruit, shells and partitions, green pericarp and leaves, bark, wood, and roots (Richter & Yadrov, 1985).

Curative and preventive properties of walnut have long been used by people in the areas of its growth – Moldova, Northern Caucasus, Romania, Tibet, Greece, Japan, China, France, etc. (Richter & Yadrov, 1985). The composition of milky-wax ripe raw walnuts includes essential oils, organic acids, alkaloids, glycosides, saponins,

coumarins, carotenoids, water-soluble vitamins, volatile, phenolic compounds, tannins, and trace elements (Yenikeeva, 2008). These natural complexes induce the therapeutic use of milky-wax ripe walnuts as supplements. The chemical composition of all parts of the walnut depends on the type, location, and environmental growing conditions.

All parts of walnut contain vitamins C, A, E, and group B, organic acids, minerals, and tannins. The unripe fruit of walnut contains up to 3–5% of vitamin C. The maximum content of vitamin C in unripe walnuts is observed at the beginning of endocarp solidification (Pilipenko & Orlova, 2009).

The main groups of minor bioactive components of food plants (quinones and hydroquinones) include juglone, which occurs in walnuts of milky-wax ripeness. Juglone (5-hydroxy-1,4-naphthoquinone) is a natural antibiotic with high bactericidal qualities. The German chemists Vogel and Reischauer first isolated it in 1856 from the green husk of walnut (Yenikeeva, 2008). Juglone inhibits the activity of *phosphatidylinositol-3-kinase,* which indicates its anticarcinogenic properties without marked toxicity inherent to other cytostatics (Babich & Stern, 1993). Juglone has high antimicrobial impact on both gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus mutans*), and gram-negative microorganisms (*Esherichia coli* and *Pseudomonas aeruginosa*), as well as on pathogenic yeast organisms (*Candida albicans*) (Polonik et al., 2004; Babula et al., 2005).

The aims of our work were to develop curd products using milky-wax ripe walnut, evaluate their chemical composition (fatty acids and amino acids), and monitor changes in sensory, physicochemical and microbiological properties of samples during storage.

MATERIALS AND METHODS

Materials

Non-fat curd, cream, starter for curd on pure cultures of lactic streptococci, calcium chloride, rennet powder, additives of fruit of walnut of milky-wax ripeness.

Additive preparation

For the production of supplements, we used different varieties of milky-wax ripe walnuts grown in the environmentally friendly Tsimlyanskii district of Rostov region (Russia). Harvesting of walnuts took place from 12 to 14 July in 2011 (average temperature was 28 ± 2°C) during fruit formation while they are rich in biologically active medicinal substances.

Honey was used as a preservative for the chopped nuts and as a sweetener. We used flower honey, which meets the requirements of the Russian standards (Russian consumer oversight agency, 2001).

Walnut additive technology consists of the following stages: receiving and sorting walnuts; washing walnuts and peeling them mechanically with a carborundum; chopping walnuts with a cutting-pulping machine, mixing chopped walnuts with honey heated to 38 ± 2°C, packaging and corking; storing at temperature 6 ± 2°C. The resulting additive is made of crushed milky-wax ripe walnuts (seed + partition + endocarp) mixed with honey; the color of the additives is homogenous and depends on the type of honey - from pale straw to dark brown; it tastes of honey and has a pronounced walnut flavour.

The preparation of curd products

Pasteurized cow cream (fat 25%) and non-fat curd were used for curd production. The cream should be homogenized (we used homogenizator Twin Panda Niro Soavi – 100 liters per hour) at temperature 60–70°C and pressure (8.0–10.0) MPa, then pasteurized at temperature 92 ± 2°C and incubated from 2 to 8 seconds, and then cooled to a temperature not exceeding 80°C (Orlova, 2007). The prepared curd and cream mixture was vacuumized and heated in a universal homogenizing module, after which it was stirred for 30 seconds at 1,500 rev min^{-1}. When the temperature reached 40 ± 2°C, the vacuum was turned off. Then we brought the supplement made of milky-wax ripe walnuts and honey. The additive was weighed, heated while mixing in the digester till 38 ± 2°C, after which it was added to the prepared curd and cream mixture. For removing air bubbles and distributing the additives uniformly, we started the stirrer and vacuum for 30 seconds. After that the product was packaged in polystyrene cups with a capacity of 200 grams. The packaged product was kept at irregular temperature for 2–3 h to prevent the formation of condensate, after which it was sent to the refrigerating chamber for cooling down till temperature 4 ± 2°C and the formation of structure for 6–8 h. The product should be stored at temperature 4 ± 2°C.

Manufacturing technology of curd products with the additive (15%) based on milky-wax ripeness walnuts is shown in Fig. 1.

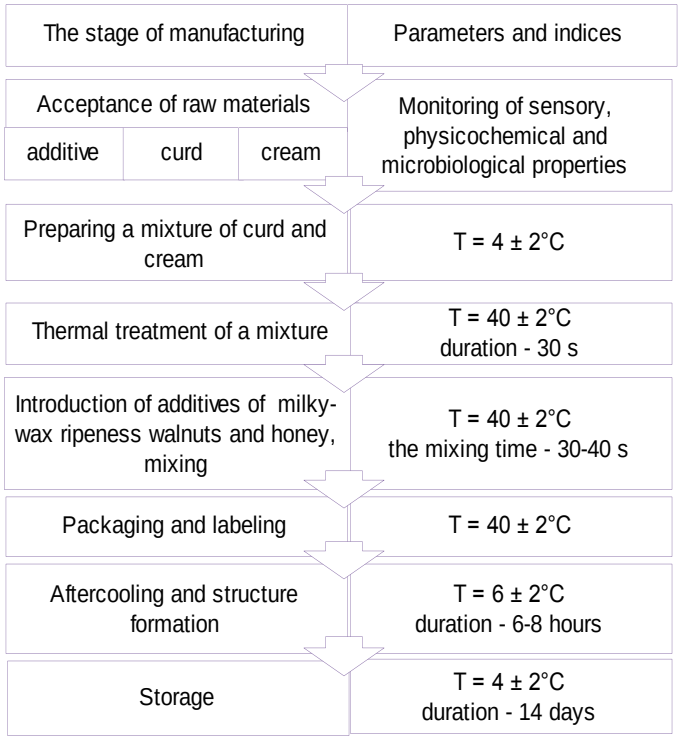

Figure 1. Curd products manufacturing technology.

Sensory evaluation

The taste, aroma, colour, and texture of all curd product samples were evaluated by a trained panel of 12 members using a five-point score system (5 excellent, 1 unacceptable).

Twelve panelists (eight women, four men; age 22–38 years) familiar with sensory evaluation techniques and regular consumers of curd products estimated the sensory properties of the samples.

Analysis

Juglone content of the walnut additives was determined by capillary electrophoresis using KAPEL 105 (sample injection 450 mbar*s, voltage 20 kV, temperature 20°C, detection 254 nm).

Amino acids, fatty acids, fat, solids, carbohydrates and titratable acidity were determined according to AOAC (1998).

Experimental design of shelf-life

Samples stored at 4 ± 2°C were evaluated on days 1, 7, 14, and 21 taking into account the factor of safety. The shelf-life of the developed products was evaluated according to MUK 4.2.1847–04 'Control methods. Biological and microbiological. Sanitary-epidemiological assessment of terms of consumption and suitability for food storage. Methodology.' (Russian consumer oversight agency, 2004).

Microbiological counts

The amount of *S. Aureus*, coliforms, yeasts and molds, and pathogens was determined by the methods described by Neusely et al., 2012.

RESULTS AND DISCUSSION

We have identified the possible amounts of walnut additives used for the developed products.

We have found out the dependence of the estimated organoleptic characteristics of the final product on the amount of contributed additive, which is expressed by the following equation 1:

$$Y = (28.026 - 0.962\ x)/(1 - 0.065\ x + 0.0013\ x^2) \quad (1)$$

Approximating function, which expresses the dependence of the organoleptic characteristics on the amount of additive and its deviation from the experimental values, is presented in Fig. 2.

The samples with 5, 10, 15% of additive had the most acceptable sensory properties. When we added more supplements, the taste of the final product was too sweet, and the consistency too liquid. The samples with the addition of less than 5% were not sweet and the taste of the additives was almost imperceptible. The resulting products had a smooth consistency without whey separation, a fermented taste of nuts and honey, and the colour varied from light cream to cream.

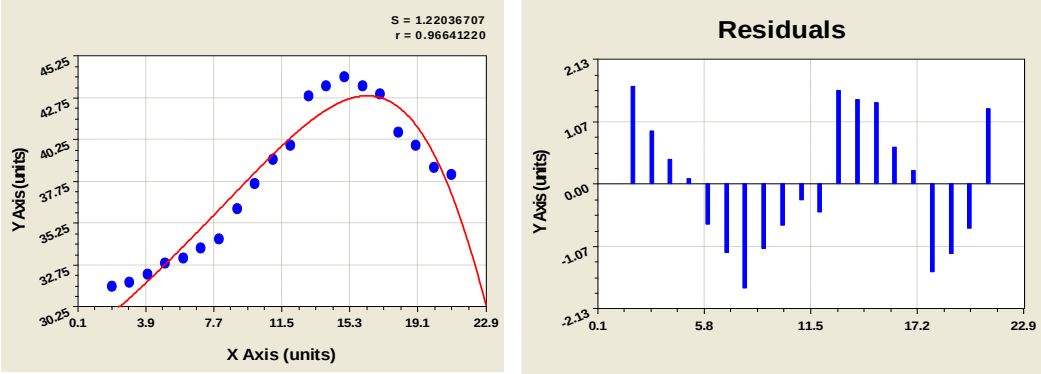

Figure 2. Approximation between organoleptic characteristics and the percentage of additives: X- the percentage of additive components, Y – organoleptic characteristics, points.

Table 1 shows the results of a study of the chemical composition of the samples of curd products. The maximum value of solids was observed in the sample with 15% additive, being 36.08%. The quantity of juglone had maximum value in the sample with 15% additive (Pilipenko & Orlova, 2009).

Table 1. Chemical composition of test samples

Sample	Mass fraction of dry substances,%	Mass fraction of protein,%	Fat mass fraction,%	Mass fraction of carbohydrates,%	Mass fraction of juglone,%
Base	36.03	14.23	10.0	11.8	0
5%	32.43	16.26	10.0	6.17	0.24
10%	34.23	15.30	10.0	8.93	0.47
15%	36.08	14.39	10.0	11.69	0.71

In order to characterize the biological value of the investigated products, the composition of amino acids was determined and the amino-acid score was calculated. The results are presented in Table 2. The results of the study of biological values indicate that the proteins of the new types of curd products are characterized by a complete set of essential amino acids. It was found out that the additive has increased the content of essential amino acids, which were especially limited in the base sample. The sample with 15% of additive had the greatest number of them. It can be explained by the fact that walnut protein has increased the content of lysine (up to 12.4 g/100 g of protein), methionine (5.6 g/100 g of protein), and tryptophan (to 3.4 g/100 g protein).

The fat of the product consisted of a mixture of milk and vegetable fats. The lipids of milk fat have 70.0% of saturated fatty acids, and about 4.0% of polyunsaturated fatty acids. The lipids of milky-wax ripe walnuts consist of 84.5% of unsaturated fatty acids, the content of linoleic and α-linolenic acid can be up to 56.0% and 11.0% respectively.

Table 2. Contents of essential amino acids in test samples

Name of amino acids	Base		5% additive		10% additive		15% additive	
	X^*, mg g^{-1}	A^*, %	X^*, mg g^{-1}	A^*, %	X^*, mg g^{-1}	A^*, %	X^*, mg g^{-1}	A^*, %
Valine	50.28	100.56	55.91	111.82	57.31	114.62	58.64	117.28
Isoleucine	48.10	120.25	54.93	137.33	57.31	143.28	59.64	149.10
Leucine	79.69	113.84	93.51	133.59	98.53	140.76	103.51	147.87
Lysine	63.77	115.95	73.67	133.95	76.77	139.58	79.82	145.13
Methionine + cystine	27.97	79.91	30.55	87.29	33.13	94.66	35.71	102.03
Threonine	36.46	91.15	50.28	125.70	60.42	151.05	70.52	176.30
Tryptophan	9.83	98.30	10.97	109.70	12.11	121.10	13.25	132.50
Phenylalanine + tyrosine	82.83	138.05	101.43	169.05	111.36	185.60	121.24	202.07

X^* is amino acid content in 1g protein product; A^* is amino-acid score of the amino acid.

The results of determining the fatty acid composition of curd products are presented in Table 3. PUFA ratio ω_6, ω_3 is 9.886 : 1, and recommended by the Institute of Nutrition, the ratio is 10 : 1 for a healthy person.

Table 3. Fatty acid composition of products

Name of acid	Fatty acid content in the test samples, g			
	Base	5% additive	10% additive	15% additive
Sum of saturated acids	**7.047**	**6.526**	**6.142**	**5.563**
Butyric	0.211	0.198	0.185	0.149
Capric	0.254	0.217	0.198	0.162
Myristic	1.384	1.354	1.347	1.331
Palmitic	3.722	3.526	3.282	3.213
Stearic	0.946	0.938	0.721	0.583
Arachic	0.090	0.070	0.056	0.049
Unidentified	0.440	0.223	0.353	0.276
Amount of unsaturated acids	**2.953**	**3.474**	**3.858**	**4.437**
Myristoleic	0.113	0.075	0.053	0.060
Palmitoleic	0.248	0.252	0.211	0.228
Oleic	2.090	2.141	2.159	2.214
Linoleic (**ω_6**)	0.189	0.698	1.124	1.523
α-linolenic (**ω_3**)	0.081	0.121	0.147	0.168
Arachidonic (**ω_6**)	0.153	0.144	0.141	0.139
Unidentified	0.078	0.043	0.023	0.105
ω_6/,ω_3	**4.275**	**7.020**	**8.687**	**9.886**

The results of organoleptic evaluation of the curd products during storage are presented in Fig. 3. The best results on the first day of storage have samples No 1 and No 4, since by all indications they have received the highest score – 20 points.

Sample No 4 got the highest score after 21 days of storage. There was no change in the appearance of the sample; the surface remained smooth and glossy with evenly distributed patches of additive. The consistency of sample No 4 was the same as that of

the fresh sample: rather viscous, thick, and slightly fluffy. The colour was cream and uniform throughout the mass, a little more intense than that of the fresh sample. The taste and smell of sample No 4 on the 21st day of storage were weaker, having a less pronounced taste of nuts and honey.

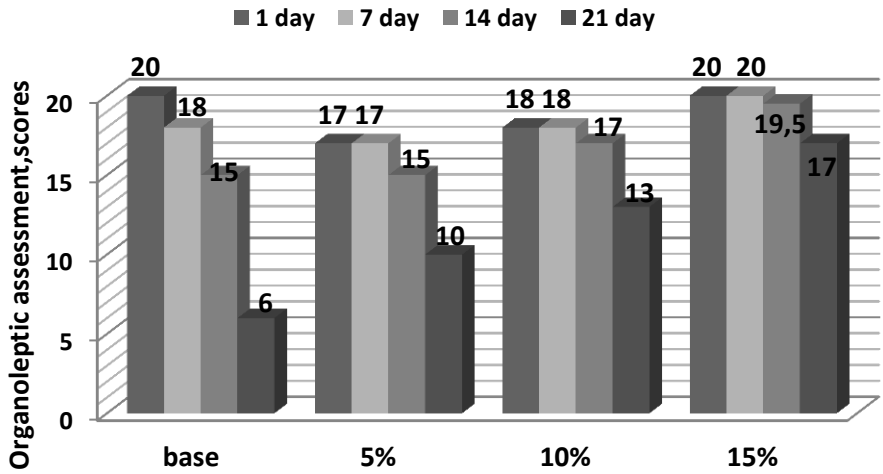

Figure 3. Organoleptic assessment during the storage of different percentages of additive components.

On the 21st day of storage, the control sample had a too acid taste and yeast flavour. Separation of serum and unpleasant odour were observed. We concluded that the product was not suitable for consumption.

The change in titratable acidity is shown in Fig. 4.

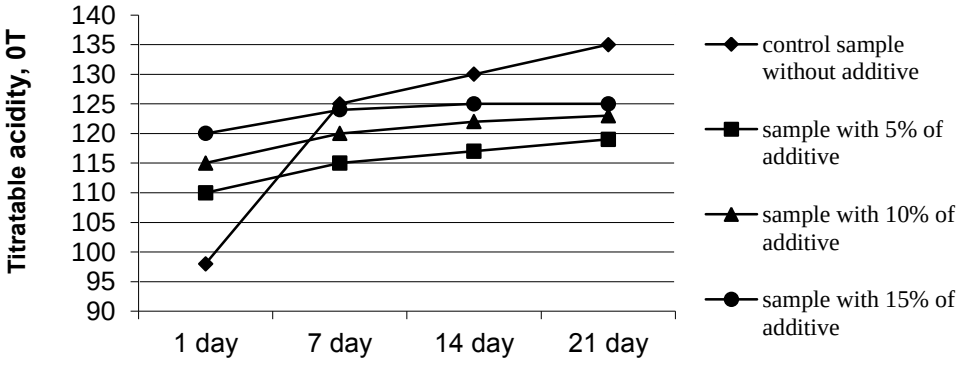

Figure 4. Change in titratable acidity of curd products during the storage.

In the control sample without the additive, quite a rapid growth of it can be observed at the beginning of the storage. After 14 days of storage, titratable acidity of the control sample did not meet the requirements of Russian legislation (Rosstandart, 2008). Introduction of supplement to the curd product allowed halting the growth of acidity in the product, especially in the sample with 15% of the additive. It is obvious that the preservative effect of juglone slows the formation of acids. It is known that juglone has properties, which partially inhibit the growth of lactic acid bacteria even in low concentrations (Babula et al., 2005).

We have investigated microbial indicators of samples in accordance with Russian legislation (Rosstandart, 2008). The results of microbiological tests (on day 14) are shown in Table 4.

In the studied samples of curd products molds, coliform bacteria in 0.001 g of product and pathogens including salmonella in 25 g were not detected. Colony-forming units (CFU) *Staph. aureus* in 1 g were not detected. The amount of yeast in the control sample did not meet the requirements of Russian legislation. Samples No 1, 2 and 3 are within the expected microbiological levels for this type of product and present no food safety concern (Rosstandart, 2008).

Table 4. The result of microbiological tests

Name of indicator	Product weight (g, cm^3), which is not allowed	Sample No1 control sample without additive	Sample No 2 sample with 5% of additive	Sample No 1 sample with 10% of additive	Sample No 1 sample with 15% of additive
CGB (coliforms), in 0.01 cm^3	0,001	not detected	not detected	not detected	not detected
S. aureus, in 0.1g	0,1	not detected	not detected	not detected	not detected
Pathogenic, including *salmonella*	25	not detected	not detected	not detected	not detected
Yeast, CFU/g	100	110	45	22	20
Molds, CFU/g	50	not detected	not detected	not detected	not detected

We assume that the reduction in yeast amount is related to the increase in additive percentage. It may be explained by the presence of naphthoquinone juglone.

CONCLUSION

Milky-wax ripe walnut is a unique product. Its amazing properties have been used since ancient times. The use of the unique characteristics of milky-wax ripe walnut (especially juglone) enables to develop fortified foods, increase the shelf life of products due to their content of naftachinon – juglone in walnuts. These studies have shown only a small part of the results of introducing walnut based additives into curd products.

Introduction of the additives allows obtaining a cream-colored product with a pronounced walnut and honey taste and odour and a smooth and pleasant consistency. The additive of milky-wax ripe walnut slows acid formation.

In contrast to the control sample, the samples with the additive displayed an increase in:

- The amount of essential amino acids, thus improving the biological value of the final product;
- The ratio of ω_6/ω_3 from 4.275 to 9.886.

The results of monitoring changes in sensory, physicochemical and microbiological properties of samples during storage allow us to recommend the shelf life of 14 days (4 ± 2°C) for curd products with additive.

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REFERENCES

AOAC. 1998. *Official Method of Analysis*. Association of Official Analytical Chemists (16th Edn), Washington DC, USA.

Al-Kadamany, E., Khattar, M., Haddad, T. & Toufeili, I. 2003. Estimation of shelf-life of concentrated yogurt by monitoring selected microbiological and physicochemical changes during storage. *Lebensm.-Wiss.U.-Technol.* **36**, 407–414.

Babich, H. & Stern, A. 1993. In vitro cytotoxicities of 1,4-naphthoquinone and hydroxylated 1,4-naphthoquinones to replicating cells. *J Appl Toxicol*. **13**(5), 353–358.

Babula, P., Mikelovab, R., Potesilb, D., Adamb, V., Kizekb, R., Haveld, L. & Sladkya ,Z. 2005. Simultaneous determination of 1,4-naphtoquinone, lawsone, juglone and plumbagin by liquid chromatography with UV detection. *Biomed. Papers* **V**, 25–28 (in Russian).

Derzhavina, N.A. 2000. *Healing walnut*. Respeks, St. Petersburg, 64pp. (in Russian).

Ji, Y., Qua, Z. & Zoua, X. 2009. Juglone-induced apoptosis in human gastric cancer SGC-7901 cells via the mitochondrial pathway. *Exp. Toxicol. Pathol.* **63**(1–2), 69–78.

Karsheva, M., Paskov, V., Tropcheva, R., Georgieva, R. & Danova, S. 2013. Physicochemical parameters and rheological properties of yoghurts during the storage. *J. Chem. Technol. Metall.* **48**, 483–488.

Liu, L., Li, W., Koike, K., Zhang, S. & Nikaido, T. 2004. New alpha-tetralonyl glucosides from the fruit of Juglans mandshurica. *Chem Pharm Bull*, **52**(5), 566–569.

Monks, T.J., Hanzlik, R.P., Cohen, G.M., Ross, D. & Graham, D.G. 1992. Quinone chemistry and toxicity. *Toxicol Appl Pharmacol*. **112**(1), 2–16.

Neusely, S., Taniwaki, M.H., Junqueira, V.C., Silveira, N., Nascimento, M. & Gomes, R.A. 2012. *Microbiological Examination Methods of Food and Water: A Laboratory Manual*, 484 pp.

Orlova, O.U. 2007. Walnuts milk-wax maturity in cheese products. *Storage and processing of agricultural* **9**, 40–42. (in Russian).

Pilipenko, T.V. & Orlova, O.U. 2009. Development of formulations of foods using raw walnut milky-wax ripeness. In Baranenko A.V.(ed.): *Collection of materials 3rd International Scientific and Technical Conference Low-Temperature and Food Technologies in XXI century*. SPbGUNiPTS St. Petersburg. pp. 290–291. (in Russian).

Polonik, S. Prokofiev, N. Agafonova, I. & Uvarov, N. 2004.The antitumor and immuno stimulatory activity O- and S-atsetilglikozidov 5-hydroxy-1,4-naphthoquinone (juglane). *Pharmaceutical Chemistry Journal* **8**, 15–17.(in Russian).

Richter, A.A. & Yadrov, A.A. 1985.*Walnut.*Agropromizdat,Moscow, 215 pp.(in Russian).

Rosstandart, 2008. Federal Law No 88 on 'Technical Regulations for Milk and Milk Products' Moscow, 124 pp.(in Russian).

Russian consumer oversight agency, 2004. MUK 4.2.1847-04 ‘Control methods. Biology and microbiological. Sanitary-epidemiological assessment of terms reasons consumption and fitness for storage of food. Methodology’, 16 pp.

Russian consumer oversight agency, 2001. Hygienic requirements for safety and nutrition value of food products. Sanitary and epidemiological rules and regulations. SanPin 2.3.2.1078-01, 180 pp.

Segura-Aguilar, J., Jonsson, K., Tidefelt, U. & Paul, C. 1992. The cytotoxic effects of 5-OH-1, 4-naphthoquinone and 5,8-diOH-1,4-naphthoquinone on doxorubicin-resis- tant human leukemia cells (HL-60). *Leuk Res*. **16**(6–7), 631–637.

Yenikeeva, R.A. 2008. *Study on Farmakognostichesky study and standardization of raw materials and preparations of walnut(Juglansregia L.).*Autoabstract, Moscow, 21 pp.

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Studying of mixing speed and temperature impacts on rheological properties of wheat flour dough using Mixolab

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Abstract. Wheat flour dough is highly non-Newtonian, time-dependent, strain-dependent and viscoelastic. These rheological properties are very sensitive to temperature, water content and composition. Dough mixing is one of the most important ways to characterize the quality of wheat flours. Proper dough development is affected by mixing intensity (mixing speed) and work imparted to the dough. The objective of this research was to study impact of mixing speed and temperature on thermomechanical properties of breadmaking quality wheat flours using Mixolab. Analysis was carried out at the constant water absorption (98% db) using standard Chopin+ protocol, which consisted of a heating/cooling cycle after a certain mixing time at constant mixing speed (60–120 rpm). Effect of temperature at 80 rpm, 100 rpm, 120 rpm, and effect of mixing speed at 30°C, 40°C, 50°C were also studied. Strong relationships were observed between the mixing speed (rpm) and the Mixolab parameters (dough consistency during mixing (C1), mixing stability, protein weakening (C2), starch gelatinization (C3), amylase activity (C4) and starch gelling (C5).
Mixing temperature was observed to have higher impact on dough consistency and stability than mixing speed. Softening effect of temperature was more significant at low mixing speeds.

Key words: mixing behavior, pasting, torque, dough consistency.

INTRODUCTION

The bread-making process consists of the three main steps. Those are mixing, fermentation and baking. The mixing process is the crucial operation in bakery product production by which the wheat flour, water, and additional ingredients are changed through the mechanical energy flow to coherent dough. (Gras et al., 2000; Zheng et al., 2000; Wilson et al., 2001). Dough mixing is one of the most important ways to characterize the quality of wheat flour samples. The dough development is a dynamic process where the viscoelastic properties are continuously changing. Therefore, dough properties are strongly influenced by the way of their mixing. For achieving the proper dough development, two basic requirements must be satisfied. The imparted mixing energy or work input must be higher than the critical limit of energy needed for gluten formation, and the mixing intensity must be above the critical level for the dough development (Kilborne & Tipples, 1972). These requirements vary with the flour

properties and the type of mixer used (Frazier et al., 1975; Oliver & Allen, 1992). For this reason, decisions with respect to adequacy of dough mixing are still partly based on operator experience. Recently, a number of analytical methods have been investigated to monitor dough development based on physical or chemical description of dough properties. The most popular in-line process measurements, based on changes in dough physical properties, are that of mixing torque or power consumption of the mixer.

At laboratory scale (using analytical methods after dough sampling), dough development has been largely investigated by microscopy and chemical analysis. In industry, a wide variety of mixing geometries and speeds are used for dough development. The way the dough is mixed has a major impact on the rheological properties due to the time- and strain-dependent nature of dough. The farinograph and mixograph are two common devices for assessing flour properties during mixing in lab scale. Both mixers provide empirical measurements related to the torque and work input required to produce optimally mixed dough, despite dissimilar geometries and mixing actions. A new generation of analytical equipment is represented by Chopin (Tripette et Renaud, Paris, France). This apparatus measures and plots in real time the torque (in N·m) produced by passage of the dough between the two mixing arms, thus allowing the study of mixing and pasting behavior of the wheat flour dough systems. Mixolab could play a key role in ensuring flour performance matches customers' expectation in finished product (Gedrovica & Karklina, 2011). The quality of wheat-hemp composites prepared with different amounts of hemp flour (5, 10, 15 and 20%) was characterised by the mixolab rheological test by Hruskova et al. The most precise distinguishing of samples was observed during the mixing and starch retrogradation phases of the test. Correlation analysis confirmed proper relationships between mixolab and rheological parameters related both to protein properties (C1, C2, C1–C2 vs. farinograph and extensigraph ones) and starch or starch gel properties (C3, C4, C5 vs. amylograph ones) (Hruskova et al., 2013). There were several research studies of thermomechanical properties of different types of wheat, such as Indian and Chines (Dhaka & Khatkar, 2013) and (Chen et al., 2013).The objective of this research was to study impact of mixing speed on thermomechanical properties of Hard Red Spring flours using Mixolab. Hard Red Spring wheat flour stands out as the aristocrat of wheat for baking bread, bagels and hard rolls. It has the highest protein content of all U.S. wheats (usually 13–16%) which, in turn, corresponds with greater gluten content in dough. Understanding the effects of mixing speed variation on gluten strength and also starch gelatinization, amylase activity and starch gelling of Hard Red Spring wheat flour is important when creating the distinct structural and textural characteristics that consumers desire in baked products.

MATERIALS AND METHODS

This study was done in the laboratory of Department of grain Science and Industry at Kansas State University.

One batch of Hard Red Spring wheat (1.4% ash, 13.2% protein, 16% moisture, 98% water absorbtion) was used for the experiments in this study during 5 consecutive days.

A standard Mixolab curves (Fig. 1) were used to determine a set of parameters listed in Table 1. C1 and C2 are related to protein quality, whereas C3, C4 and C5 are related to the starch characteristics. Fig.1 shows the results of experiment No 3 (Table 2). Correlations between mixing speed and the mixolab parameters and also the correlations between initial bowl temperature and mixolab parameters were investigated.

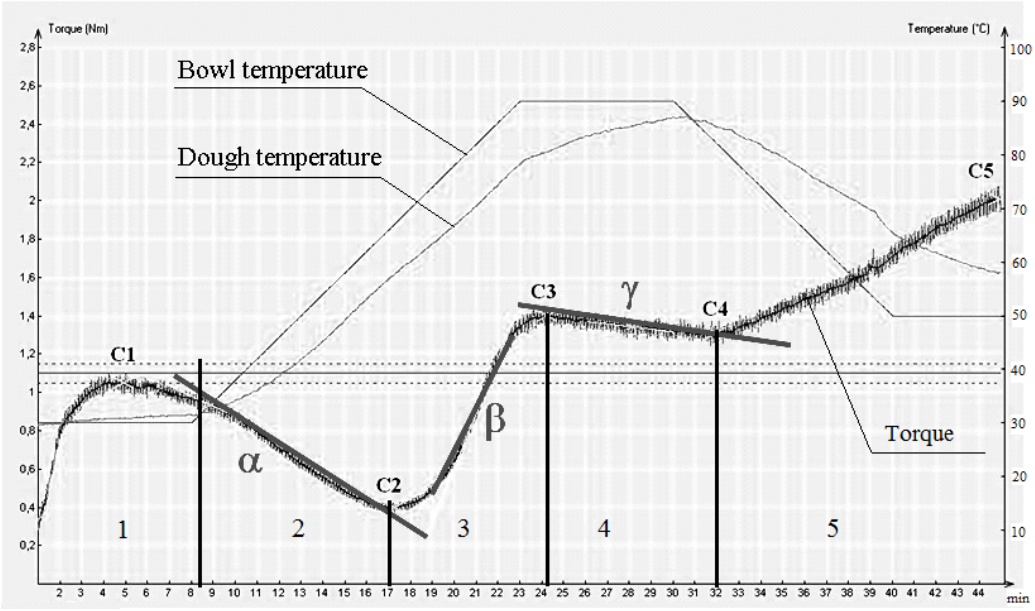

Figure 1. Mixolab Chopin+ protocol curve. Experiment No 3 (80 rpm and 30°C); where: α, β, and γ are the indicators of protein weakening, starching speed and enzymatic degradation.
Zone 1: Dough Development – at constant temperature, the start of the test determines the water absorption capacity of the flours and measures the characteristics of dough during mixing (stability, elasticity, absorbed power); Zone 2: Protein reduction (α) – when dough temperature increases, consistency decreases. The intensity of this decrease depends on protein quality; Zone 3: Starch gelatinisation (β) – as from a certain temperature, the phenomena linked to starch gelatinisation become dominant and an increase in consistency is then observed. The intensity of this increase depends on the quality of the starch and, in some cases, on the additives; Zone 4: Amylase activity (γ) – The value of consistency at the end of the plateau depends considerably on the endogenous or added amylasic activity. The greater the decrease in consistency, the greater the amylasic activity.

The Mixolab is a recording dough mixer used to measure the rheological properties of doughs subject to the dual stress of mixing and temperature changes. It measures the torque (in N·m) produced by the dough between two mixing blades. The test is based on the preparation of a constant dough sample weight hydrated to obtain a target consistency during the first test phase. In the 'Chopin+' protocol, the dough weight is 75 grams and the target consistency is 1.1 N·m (± 0.05 Nm).

Mixolab analysis were carried out at the constant water absorption (98% db) using standard 'Chopin+' protocol, which consisted of a heating/cooling cycle after a certain mixing time at constant mixing speed (60–120 rpm). Required amount of flour for analysis was calculated by Mixolab software according to input values of flour mixtures moisture as well as water absorption. The total mass of flour and distilled water placed into bowl was 75 g. Initial bowl temperature for each experiment is shown in Table 2.

Table 1. Mixolab parameters

Point	Description
C1	Maximum consistancy obtained in the first 8 min (water absorbtion)
C2	Protein weakening as a function of mechanical work and temperature
C3	Starch gelatinisation
C4	Hot gel stability
C5	Starch retrogradation in the cooling phase
Slope α – slope of curve between end of period at 30°C and C2	Protein weakning speed under the effect of heat
Slope β – Slope of curve between C2 and C3	Starch gelatinisation speed
Slope γ – Slope of curve between C3 and C4	Enzyme degradation speed

Table 2. Experiment composition (target torque for C1 – 1.1 N·m)

Experiment	Number of experiment	Mixing speed, rpm	Initial bowl and 1-st step temperature, °C
Speed effect study	1	60	30
	2	70	30
	3	80	30
	4	90	30
	5	100	30
	6	110	30
	7	120	30
Temperature and speed effects study	8	80	40
	9	80	50
	10	100	40
	11	100	50
	12	120	40
	13	120	50

After dough mixing stage (8 minutes) samples temperature increase with the speed 4°C min^{-1} during 15 minutes; at this point, there was a holding period for 7 minutes at 90°C, followed by a temperature decrease with the speed 4°C per min during 10 minutes; then the mixture reached 50°C and hold at this temperature for 5 minutes. Total analysis time was 45 min. The mixing speed during the entire assay from very beginning until the end was 60, 70, 80, 90, 100, 110 and 120 rpm, respectively to the experiment. 5 replicates were carried out for each type of experiments.

RESULTS AND DISCUSSION

Initial testing has focused on mixing flour-water dough to peak development at varying speeds (Fig. 2). Work input to reach peak torque was determined and compared (Pastukhov & Dogan, 2010).

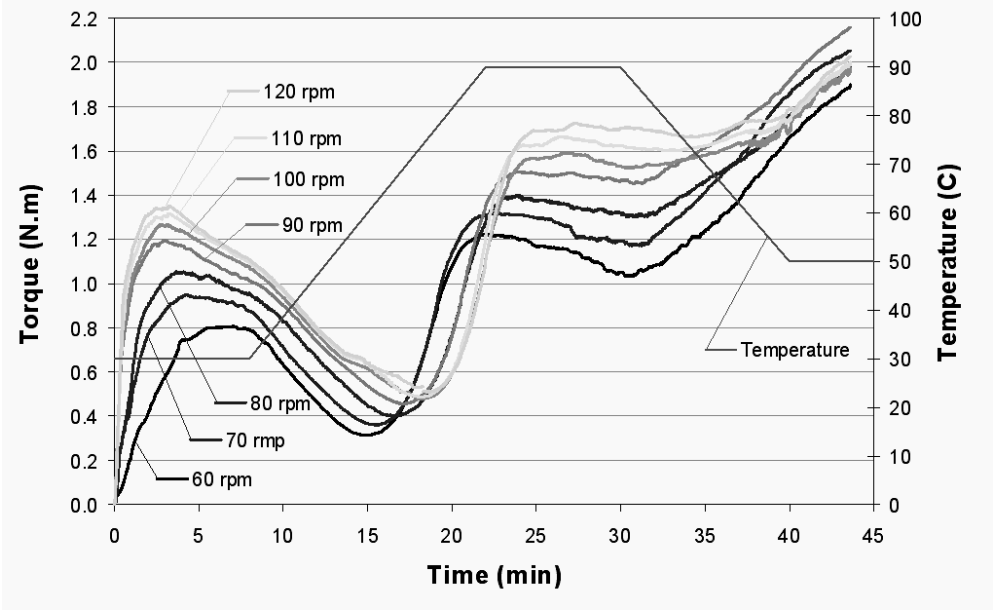

Figure 2. Mixolab curves obtained at varying mixing speeds (60–120 rpm) using 'Chopin+' protocol (Speed effect study).

Strong relationships were observed between the mixing speed (rpm) and the following Mixolab parameters: (Fig. 3) a) time needed to reach point C1; b) torque in point C1; c) torque in point C2; d) C1–C2 difference indicating progressive protein weakening; e) torque in point C3; f) torque in point C4; g) C3–C4 difference indicating starch stabilization; h) α; i) total work done (sum of the torques during the experiment).

It is known from (Sabovics et al., 2011) that decrease of triticale flour proportion in blend during mixing with constant speed results to increasing of the dough stability and does not change dough properties substantially. Changing the mixing speed we discovered that dough consistency increased while the stability decreased with increasing mixing speed (Fig. 2, Fig. 3). The higher the mixing speed the faster the achievement of point C1 takes place and the higher the torque in this point (Fig. 3 a, b). The same situation with torque in points C2, C3, C4, but the time needed to reach these points are increasing with increasing of mixing speed. C2–C1 difference increased indicating progressive weakening in dough network at elevated mechanical energy input and temperature. Maximum viscosity (point C3) increased possibly due to quick rupture of starch granules leading to lower pasting temperatures and to higher paste consistency. C3–C4 difference – fall in viscosity (stability when hot) is decreasing when mixing speed is increased. Value of α slope increases monotonically with increasing of mixing speed, indicating the protein weakening (Fig. 3 h).

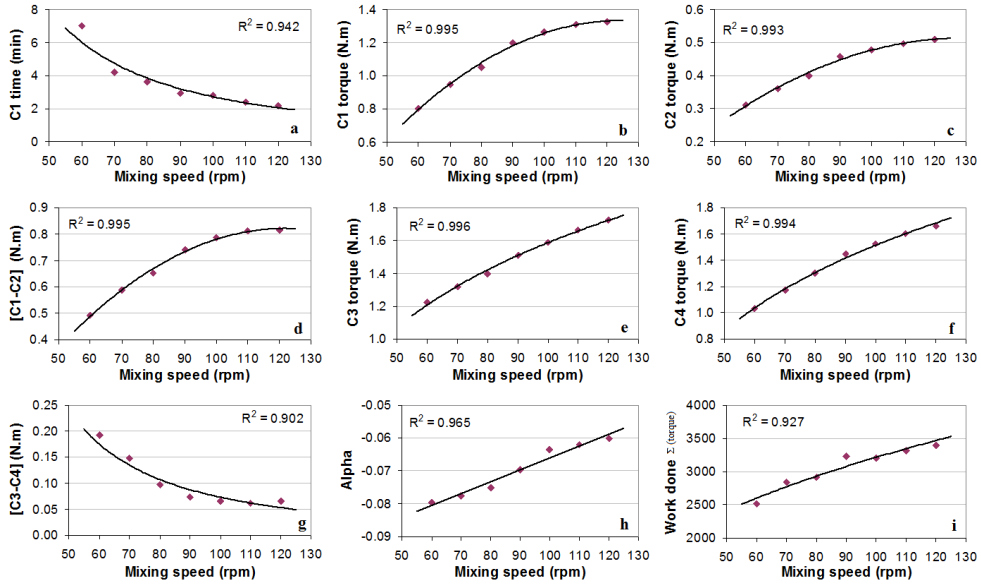

Figure 3. Correlations between mixing speed and Mixolab parameters.

Fig. 4 shows the effect of temperature at 80 rpm (a), 100 rpm (b), 120 rpm (c), and effect of mixing speed at 30°C (d), 40°C (e), 50°C (f) observed in the second set of experiments

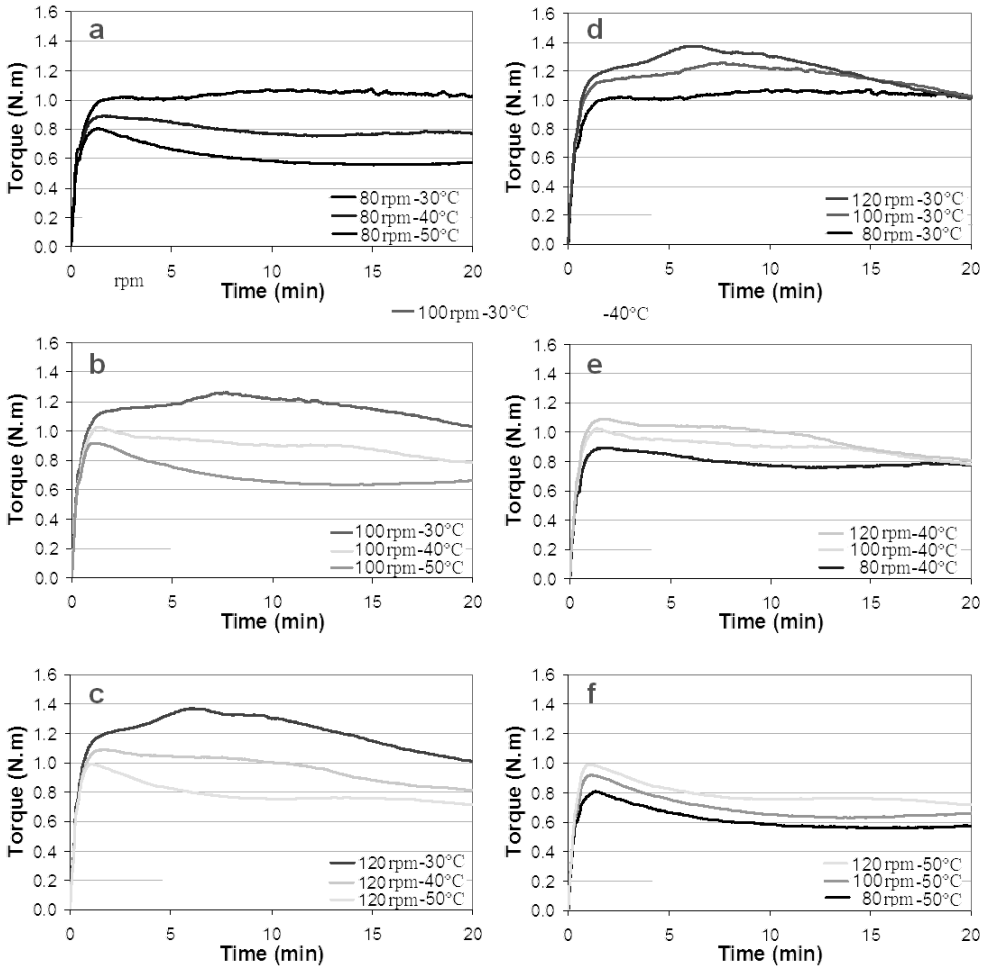

Figure 4. Effect of temperature at a – 80 rpm, b – 100 rpm, c – 120 rpm, and effect of mixing speed at d – 30°C, e – 40°C, f – 50°C.

CONCLUSIONS

Results indicated that the speed at which dough is deformed during mixing can cause it to develop differently.

Dough development time decreased significantly with gradual increase in mixing speed.

Stability of gluten network dropped sharply as mixing speed increased as indicated by C1–C2 and α (slope of the descending curve) values.

Increase in mixing speed resulted in increased higher dough consistency independent from the mixing temperature. Mixing temperature was observed to have higher impact on dough consistency and stability than mixing speed. Softening effect of temperature was more significant at low mixing speeds. The present study showed that Mixolab has ability to easily model different speed variations and results of these experiments indicate that the speed at which dough is deformed during mixing can

cause it to develop differently. However, further work is required for modeling more complicated mechanical motion of mixing arms as we can meet in real mixers. It can be concluded that Mixolab is a suitable instrument for progressive work in scientific laboratories and industrial bakeries.

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REFERENCES

Chen, F., Li, H., Li, X., Dong, Z., Zuo, A., Shang, X. & Cui, D. 2013. Alveograph and Mixolab parameters associated with Puroindoline-D1 genes in Chinese winter wheats. *J. Sci. Food Agric.* **93**(10), 2541–2548.

Dhaka, V. & Khatkar, B.S. 2013. Mixolab thermomechanical characteristics of dough and bread making quality of Indian wheat varieties. *Qual. Assur. Saf. Crop.* **5**(4), 311–323.

Gedrovica, I. & Karklina, D. 2011. Influence of Jerusalem artichoke powder on dough rheological properties. *Proceedings of the 6th Baltic Conference on Food Science and Technology 'Innovations for Food Science and Production' FOODBALT-2011*, Jelgava, May 5–6, pp.7–12.

Gras, P.W., Carpenter, H.C. & Andersen, R.S. 2000. Modelling the developmental rheology of wheat flour dough using extension tests. *J. Cereal Sci.* **31**, 1–13.

Hruskova, M., Svec, I. & Jurinova, I. 2013. Changes in Baking Quality of Composite Wheat/Hemp Flour Detected by Means of Mixolab. *Cereal Res. Commun.* **41**(1), 150–159.

Kilborne, R.H. & Tipples, K.H. 1972. Factors affecting mechanical dough development. I. Effect of mixing intensity and work input. *Cereal Chem.* **49**, 48–53.

Olivier, J.R. & Allen, H.M. 1992. The prediction of breadmaking performance using the farinograph and extensograph. *J. Cereal Sci.* **15**, 79–89.

Pastukhov, A.S. & Dogan, H. 2010. Effect of kneading speed on thermo mechanical properties of flour doughs. Kansas State University, Manhattan, KS, U.S.A. *Cereal Foods World*, **55**: A63

Sabovics, M., Straumite, E. & Galoburda R. 2011. Assessment of the rheological properties of flour using the mixolab. *Proceedings of the 6th Baltic Conference on Food Science and Technology 'Innovations for Food Science and Production' FOODBALT-2011*, Jelgava, May 5–6, pp.33–38.

Wilson, A.J., Morgenstern, M.P. & Kavale, S. 2001. Mixing response of a variable speed 125 g laboratory scale mechanical dough development mixer. *J. Cereal Sci.* **34,** 151–158.

Zheng, H., Morgenstern, M.P., Campanella, O.H. & Larsen, N.G. 2000. Rheological properties of dough during mechanical dough development. *J. Cereal Sci.* **32**, 293–306.

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Effect of cooling rates and low crystallization temperatures on morphology of lactose crystals obtained from Ricotta cheese whey

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Abstract. In the traditional process of lactose production from cheese whey crystallization temperature is reached by slow cooling. Lactose morphology obtained in this way has been well investigated. The objective of this work was to study morphological parameters of lactose crystals during crystallization at low temperatures, using rapid and extra rapid cooling. Ricotta whey was chosen for analysis because this raw material has been poorly investigated as a source of lactose production. Three temperatures (2, 6 and 12 ℃) were used for crystallization of lactose. Rapid (0.5°C min^{-1}) and extra rapid cooling (3°C min^{-1}) rates were used to achieve these temperatures. Dimensions of crystals were examined with optical stereo-, and scanning electron microscopes. Pure lactose solution was used as a reference during the study. The results of the study showed the impact of some Ricotta whey components on the crystals morphology and size. These components reduce crystals size, and linked with low crystallization temperature, modify the shape of crystals faces. Extra rapid cooling of Ricotta whey contributes to the growth of needle-like crystals more than the rapid one. In a pure lactose solution no needle-shaped crystals were observed.

Key words: lactose, Ricotta cheese whey, crystallization, cooling rate.

INTRODUCTION

Industrial crystallization of lactose from cheese whey is a slow process based on cooling of deproteinated and concentrated whey. First of all, a concentrate is cooled rapidly to 30°C. After that it is seeded and cooled rather slowly, at about 1 to 3°C h^{-1}. Crystallization temperature of 20°C is normally recommended. (Tan, 2010) As early as in 1930s, Whritter & Could (1931) proposed such scheme for lactose production because they found that lactose crystallization was faster at 30°C than at any lower temperature. Crystallization of lactose at low temperatures has been well studied over many decades in connection with ice-cream investigations (Nickerson, 1954, 1956; Livney et al., 1995). The phenomenon of cooling rate in general has also been investigated and used for manufacturing of lactose (Paterson, 2010). No information was found in the literature concerning the effects of low crystallization temperatures and the cooling rate on the morphological parameters of lactose crystals. The objective of this work was to study morphological parameters of lactose crystals during crystallization at low temperatures, using rapid and extra rapid cooling.

MATERIALS AND METHODS

Ricotta cheese whey was used for this study because of additional deproteinization process carried out during Ricotta production. It was provided by a local cheese factory, OÜ Põltsamaa Juustutööstus, Estonia. The lactose, fat and protein contents were measured at the Milk Analysis Laboratory of the Estonian Animal Recording Centre using an automated infrared milk analyzer (CombiFoss FT+, Foss Electric, Denmark). The ash content was estimated using the International IDF Standard 42B : 1990; pH was measured by the METTLER TOLEDO pH-meter (Mettler-Toledo International Inc., Switzerland). The dry matter content was measured using METTLER TOLEDO RH83 moisture analyzer (Mettler-Toledo International Inc., Switzerland).

Ricotta whey was heated to 90°C and then 0.4% w/w of lactic acid (Sigma-Aldrich Chemie GmbH, Germany) was added for precipitation of protein residues, which were removed by filtering through common paper filter. Concentration of Ricotta cheese whey was carried out by water evaporation at low temperature (60°C) during approximately 3.5 h. Concentration factor of 10 was achieved by this treatment. It corresponds to total solids concentration of ca 50% w/w (Pisponen et al., 2013). Concentrated Ricotta whey was poured into test tubes, by 10 ml into each one. Crystallization was carried out at the temperatures of 2, 6 and 12°C during 24 hours without agitation in an incubator Panasonic MIR-154-PE (Japan). Rapid (0.5°C min^{-1}) and extra rapid cooling (3°C min^{-1}) rates were used to achieve these temperatures. Extra rapid cooling was carried out using ultra low temperature freezer Panasonic MDF-C8V1 (Japan). The pure lactose solution with concentration 50% w/w was used as reference solution and was subjected to the same stages, in order to study the impact of impurities on crystallization process. Thus, there were two investigated parameters for each sample: cooling rate and temperature of crystallization. Crystals were washed with 10% w/w spirit solution and distilled water to clean crystals from molasses.

The shape and the size of crystals were examined using a microscope Nikon SMZ 1000 (Nikon Corporation, Japan), equipped with the digital camera Nikon DS-U2/L2 USB (Nikon Corporation, Japan). The height of crystals was estimated using the software NIS-Elements D 3.1 (Nikon Corporation, Japan). Used measurement technique has been described in our earlier study in Pisponen et al. (2013). At least 40 crystals were examined for each data set. In order to avoid errors, only single, clearly distinguishable crystals were inspected. A scanning electron microscope Leo 1430VP (LEO Electron Microscopy Ltd, England) was used for studying the surface of crystals.

RESULTS AND DISCUSSION

The content of dry matter, fat, protein and lactose in Ricotta cheese whey are given in Table 1. Five measurements were considered for each data set. Data values obtained in this study were lower than those of the normal cheese whey (Jelen, 2003).

The average values of crystals heights are shown in Table 2. Crystals obtained from pure lactose solution in most cases were larger than crystals obtained from Ricotta cheese whey. This difference can be explained by presence of protein residues in Ricotta cheese whey. Modler & Lefkovitch (1986) and Miumoni et al. (2005)

demonstrated in their research with cheese whey that protein lowered the final size of crystals. Minerals, contained in Ricotta cheese whey, can also lower the final size of crystals. Guu & Zall (1991) found, that potassium, sodium and calcium can retard or inhibit lactose crystallization from cheese whey. General appearance of crystals is shown in Fig. 1.

Table 1. Content of dry matter, fat, protein, lactose, ash content (%w/w) and pH in native Ricotta cheese whey

	Dry matter	Fat	Protein	Lactose	pH	Ash content
Average	4.9	0.16	0.44	3.85	5.67	0.51
Standard deviation	0.23	0.01	0.02	0.07	0.08	0.08

Table 2. The average, ± standard deviation, minimum and maximum values (within brackets) of crystals height (μm) obtained from different solutions under different crystallization temperatures and cooling rates

Temperature,°C	2		6		12	
Rate of cooling	Rapid	Extra rapid	Rapid	Extra rapid	Rapid	Extra rapid
Ricotta cheese whey	155 ± 20 (128–196)	78 ± 26 (41–127)	182 ± 76 (93–388)	133 ± 73 (39–384)	179 ± 59 (80–351)	123 ± 53 (62–337)
Pure lactose solution	166 ± 77 (77–450)	128 ± 52 (39–288)	165 ± 66 (46–302)	192 ± 72 (94–385)	158 ± 68 (55–387)	220 ± 65 (111–372)

Rapid cooling does not affect the size of crystals obtained from pure lactose solution; they remained almost identical at different crystallization temperatures. When using extra rapid cooling, the difference in crystals sizes became obvious. The height of crystals, obtained from pure lactose solution, increased along with rising of crystallization temperatures. There was no such a clear dependence in size of crystals, obtained from Ricotta cheese whey; the shortest crystals appeared at 2°C and the highest at 6°C. Difference in sizes between crystals, obtained at 6 and 12°C, was considerably smaller than between 2 and 6°C. This indicates to a certain optimum between 2 and 12°C. A large amount of needle-shape crystals was obtained at 6 and 12°C during crystallization from Ricotta cheese whey at extra rapid cooling (Fig. 1). Needle-shaped crystals are formed when the super saturation of material in solution is very high (Hartel, 2001). Extra rapid cooling increases the content of crystalline material in solution and promotes the appearance of a host in crystallization centers (Paterson, 2010). In our experiment, extra rapid cooling could promote occurrence of even more crystallization centers, which lead to appearance of extra small crystals. There were no needle-shaped crystals observed in a pure lactose solution (Fig 1), which leads to the conclusion that impurities have bigger impact on the crystal form than crystallization conditions. Crystals obtained in the research were generally smaller than crystals obtained in our earlier study at the same concentration factor, but at a slower cooling rate (Pisponen et al., 2013).

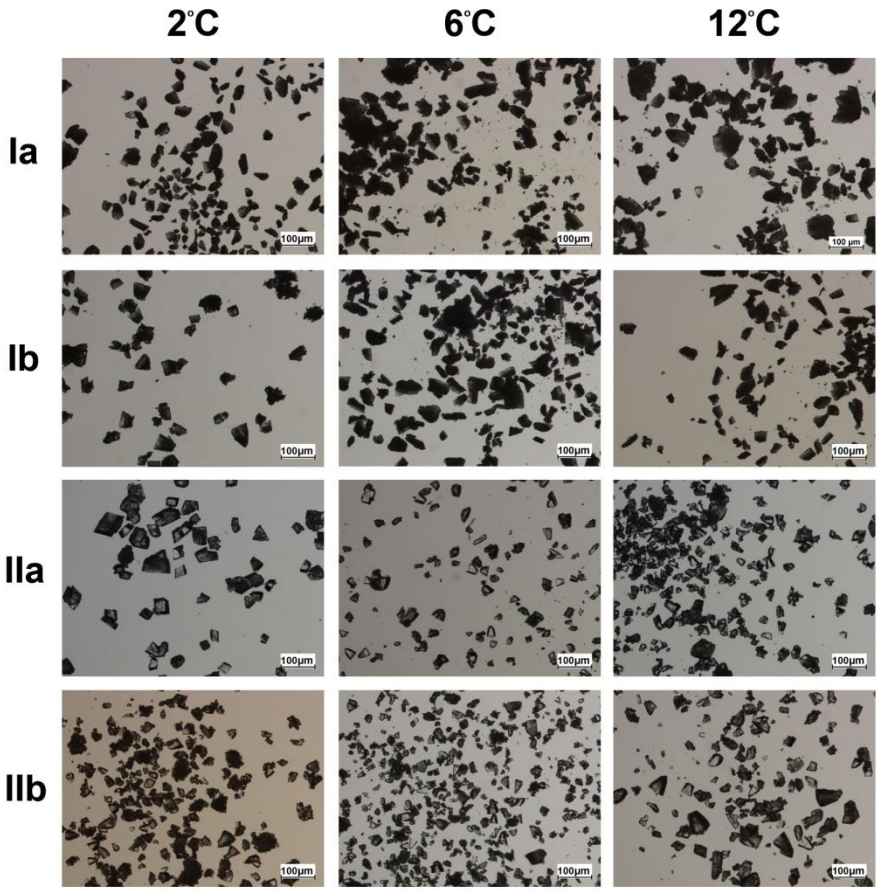

Figure 1. Lactose crystals forms depending on crystallization temperature and cooling rate: solution: I–Ricotta cheese whey II–pure lactose solution: a–rapid cooling and b–extra rapid cooling.

The SEM photos show that faces of some crystals, grown in Ricotta cheese whey, were modified (Fig. 2 b). According to Hartel`s (2001) theory crystals grow imperfectly, if their growth speed is too rapid. Molecules do not have time to attach the right place on the crystal face (in the crystal lattice). They rapidly incorporate at random position, making crystal surface rough. In our case crystals were growing very rapidly due to a sharp drop in temperature. But crystals, grown at the same conditions in pure lactose solution, had smooth faces (Fig. 2 a).

It can be supposed, that not only growth speed can affect the shape of crystals. Macromolecules from Ricotta cheese whey such as proteins may adsorb on the crystal surface and influence the manner by which molecules attach to the crystals lattice (Hartel & Shastry, 1991). Riboflavin can also adsorb on the growing crystal and modify its shape (Holsinger, 1988). Lifran et al. (2007) found, that riboflavin makes the tip of the crystal irregular, which can be observed on crystals, obtained from Ricotta cheese whey (Fig. 2 b).

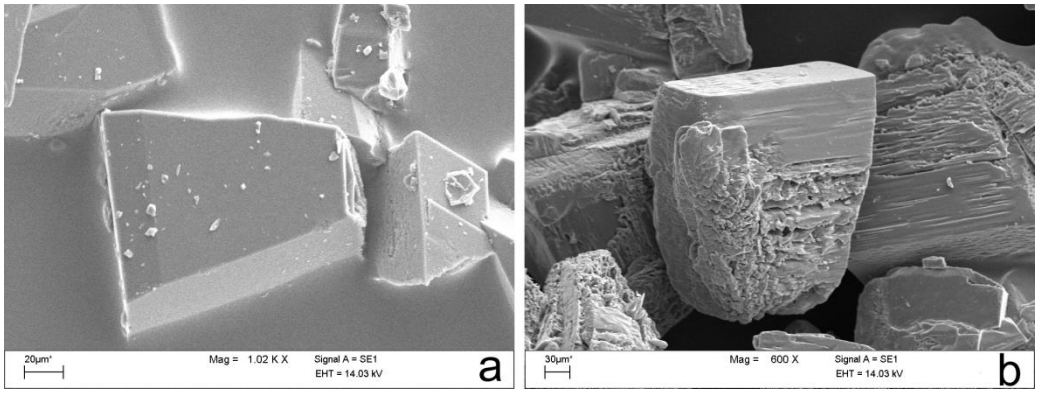

Figure 2. SEM images of lactose growing in a – pure supersaturated lactose solution, b – Ricotta cheese whey.

According to Cabrera-Vermilyea (1958) model, impurities adsorbed on the crystal face prevent the smooth growth of a new layer. Crystal of lactose grows in a spiral (Dincer et al., 2009); therefore layers grow one upon another. If the lower layer`s growth is stopped by immobile impurity, the upper layer grows to it, until it completely covers impurity and move farther (Hartel, 2001). Any kind of particles adsorbed on a crystal face, or rough and pitted surface, can potentially serve as nucleation centers. Furthermore, a rapid rate of cooling generally leads to nucleation at a lower temperature, rather than the slow cooling. (Hartel, 2001) In our case, these effects could trigger nucleation on the crystal surface and increase its roughness. However, it contradicts the theory that under conditions of rapid growth the additives cannot adsorb on the surface because of competition with the molecules of crystallizing material (Holsinger, 1988).

It remains unclear why (010) faces (base of crystal) were well-developed when other faces were rough (Fig. 2 b). Inasmuch as (010) face becomes the fastest growing face (Dincer et al., 2009), it can be assumed that it starts to form until the supersaturation becomes critical and impurities from solution accumulates in the boundary layer of the crystal later on (Hartel & Shastry, 1991).

CONCLUSIONS

This study presents influence of rapid and extra rapid cooling rate and crystallization at low temperatures on the morphological parameters of lactose crystals. Experiments show that temperature of crystallization and cooling rate impact mainly the size of lactose crystals. A strong dependence between crystallization temperature, cooling rate and crystals sizes was observed. Morphology of crystals was affected by impurities from solution more than crystallization conditions. There was some amount of irregular and needle-shaped crystals, obtained from Ricotta cheese whey. In a pure lactose solution no needle-shaped crystals were observed. The impact of impurities on certain faces of crystal requires an additional research.

REFERENCES

Cabrera, N. & Vermilyea, D.A. 1958. The Growth of Crystal from Solution. In Doremus, R.H., Roberts, B.W. & Turnbull, D. (eds.): *Growth and Perfection of Crystals.* Chapman & Hall, London, pp. 393–410.

Dincer, T.D., Ogden, M.I. & Parkinson, G.M. 2009. Crystal growth mechanisms of the (010) face of alpha-lactose monohydrate crystals. *J. Crys. Gro*. **311**, 2427–2432.

Guu, M.Y.K. & Zall, R.R. 1991. Lactose crystallization: Effects of minerals and seeding. *Process Biochem.* **26**, 167–172.

Hartel, R.W. 2001. *Crystallization in Foods*, Aspen Publishers, Inc, Gaithersburg, 325 pp.

Hartel, R.W. & Shastry, A.V. 1991. Sugar crystallization in food products. *Crit. Rev. Food Sci. & Nutr.* **1**(1), 49–112.

Holsinger, V.H. 1988. Lactose. In Jennes, R., Marth, E.H., Wong, N.P., Keeney, M. (eds): *Fundamentals of Dairy Chemistry, 3rd edn*, Springer, New York, pp. 279–343.

Jelen, P. 2003. Whey: Composition, properties, processing and uses. In Roginski, H. (ed): *Encyclopedia of Dairy Science.* Academic Press, Waltham, pp. 2653–2661.

Lifran, E.V., Vu, T.T.L., Durham, R.J., Hourigan, J.A. & Sleigh, R.W. 2007. Crystallization kinetics of lactose in the presence of lactose phosphate. *Powder Tech*. **179**, 43–54.

Livney, Y.D., Donhowe, D.P. & Hartel, R.W. 1995. Influence of temperature on crystallization of lactose in ice-cream. *Int. J. Food Sci. Tech.* 30, 311–320.

Miumoni, A., Schuck, P. & Bouhallab, S. 2005. Kinetics of lactose crystallization and crystal size as monitored by refractometry and laser light scattering: effect of proteins. *Dairy Sci. Tech* **85**, 253–260.

Modler, H.W. & Lefkovitch, L.P. 1986. Influence of pH, casein and whey protein denaturation on the composition, crystal size nad yield of lactose from condensed whey. *J. Dairy Sci.* **69**, 684–697.

Nickerson, T.A. 1956. Lactose crystallization in ice cream II. Factors affecting rate and quantity. *J. Dairy Sci.* **39**(10), 1342–1350.

Nickerson, T.A. 1954. Lactose crystallization in ice cream I. Control of crystal size by seeding. *J. Dairy Sci.* **37**(9), 1099–1105.

Paterson, AH.J. 2010. Production and Uses of Lactose. In McSweeney, P.L.H. & Fox, P.F. (eds): *Advanced in Dairy Chemistry Volume 3: Lactose, Water, Salts and Minor Constituents, 3rd edn*, Springer, New York, pp. 105–120.

Pisponen, A., Pajumägi, S., Mootse, H., Karus, A. & Poikalainen, V. 2013. The lactose from Ricotta cheese whey: the effect of pH and concentration on the size and morphology of lactose crystals. *Dairy Sci. & Technol.* **93**, 477–486.

Tan, R. 2010. Manufacture of Sweetened Condenced Milk and the Significance of Lactose Therein. In McSweeney, P.L.H., Fox, P.F. (eds): *Advanced in Dairy Chemistry Volume 3: Lactose, Water, Salts and Minor Constituents, 3rd edn*, Springer, New York, pp. 36–57.

Whritten, E.O. & Gould, S.P. 1931. Speed of crystallization of lactose, galactose, glucose and sucrose from pure solution. *Ind. Eng. Chem.* **23**(6), 670–673.

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An alternative method for meat shear energy estimation during ageing

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Abstract. The aim of this research was to study an alternative method (gravitational impulse method) for meat shear energy estimation. A falling shear blade (guillotine) with constant potential energy was used. Shear energy was determined as the difference between the initial potential energy of the blade and the residual energy measured via the impulse provided by the shear blade during collision with the force plate on the bottom of the device. The shear energy measured by the gravitational impulse method was compared to the data received by the texture analyser TMS PRO based on the Warner-Bratzler method. The meat shear parameters of de-boned beef striploin samples were measured after 2, 7, 14, 21, 28 and 35 days of ageing at 0–2°C. The results of both methods (gravitational impulse method and Warner-Bratzler method) indicated similar trends of shear energy lessening during beef ageing. Based on the preliminary results, it can be concluded that the gravitational impulse method is suitable for evaluation of meat texture during ageing as well. The main advantage of this method is its simplicity and the low price of the device.

Key words: Warner-Bratzler share force test, gravitational impulse method, beef shear energy.

Abbreviation key: MLD – *Musculus longissimus dorsi*, WB – Warner-Bratzler, GIM – gravitational impulse method.

INTRODUCTION

Beef texture is tough, thus it must be aged before consumption to improve the meat tenderness (Field et al., 1971; Jennings et al., 1978; Mottram, 1998). Textural parameters are very important during meat processing and regarding the sensory characteristics of meat products. Special texture analyzers are produced for quantitative evaluation of meat texture, which measure force dynamics at constant cutting speed. Such texture analyzers based on the Warner-Bratzler (WB) methodology and with determined characteristics are mainly used in scientific investigations (Wheeler et al., 1999; Hopkins et al., 2011). Application of these devices in meat industry is not common due to their operational complexity and considerably high price.

The aim of this work was development and evaluation of an alternative device based on the gravitational impulse method (GIM) for estimation of beef shear energy during ageing. Shear energy of aged steak samples was measured by the gravitational impulse method (GIM) and with the TMS PRO equipment based on the Warner-Bratzler method (Tommy et al., 2013) during the study.

MATERIALS AND METHODS

An experimental device using the gravitational impulse method (GIM) for estimation of meat shear energy was developed at the Department of Food Science and Technology of the Estonian University of Life Sciences. The GIM device contains a blade, a cutting table and a force plate with a transducer to record impulses (Fig 1). To carry out measurements, a meat sample is placed on the cutting table between the shear blade and force plate, where the sample is penetrated by the free fall of the blade. After shredding the sample, the blade falls onto the force plate and its transducer generates an impulse, which is recorded by a measurement controller. The weight of the blade is 1.10 kg and its initial height from the force plate is 460 mm. The shear energy can be determined as a difference between the initial potential energy of the blade and the residual energy generating an impulse on the force plate. The softer the structure of the meat, the stronger the strike to the force transducer and vice versa.

Figure 1. The test device to determine the shear energy of meat by using GIM method (1 – blade, 2 – force transducer, 3 – blade leading rods, 4 – slidebearing, 5 – cutting table).

Physics of the GIM device based on energy balance. If the blade is lifted to a certain altitude (h), it holds the potential energy (E_P).

While dropping the blade from that height, the potential energy (E_P) turns into kinetic energy (E_K), the end value of which corresponds to the size of the impulse (i) generated by the force plate transducer 1 (Fig. 2). This can be estimated by calculation of the surface area under the graph of force dynamics. The meat sample on cutting table will consume a part of that energy (shear energy E_L), and the force transducer will record a lower result accordingly.

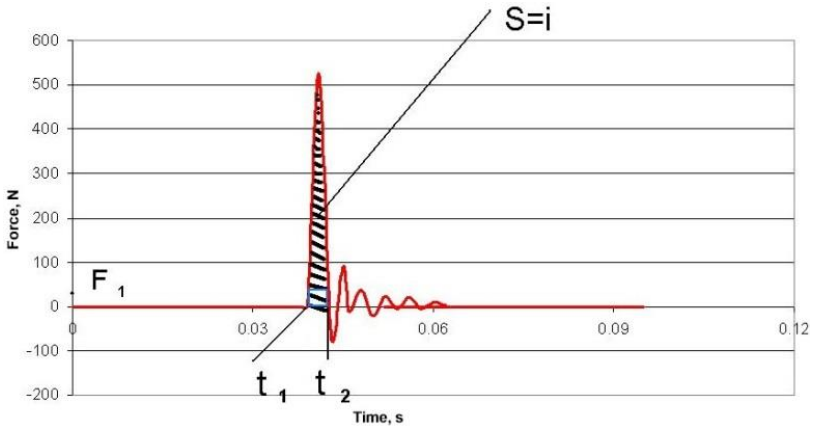

Figure 2. Example of the force dynamics recorded by the force plate transducer of the GIM device during a measurement cycle.

Experiments were carried out at the Department of Food Science and Technology (Estonian University of Life Sciences) in 2013. Three deboned *M. longissimus dorsi* muscles (MLD) removed from beef carcasses two days after slaughtering were used for the experiments. Each muscle was cut into six samples with the weight of about 300 g each and aged in vacuum packages at 0–2°C for 2, 7, 14, 21, 38 and 35 days. Hanzelková et al. (2011) have suggested that for improvement of beef tenderness it should be aged for at least 14 days. The total ageing time in our investigation was chosen to cover all spans reported in the literature on different meat ageing studies (Koohmaraie et al., 1995; Sañudos et al., 2004; Vieira et al., 2007; Muchenje et al., 2008). Preparation of probes for analyses was carried out according to the methodology of WB (Savell et al., 2013). Probes were obtained from the meat samples by using a hollow drill with the inner diameter of 20 mm. Up to ten probes were drilled off each of the MLD segments along the muscle fibres (Fig. 3). The shear force dynamics of both raw and thermally treated samples of aged beef were registered by the WB and GIM methods. Later, total shear energy was calculated from force dynamics. Probes were sheared from the middle perpendicularly to the muscle fibres in six separate trials. For thermal treatment, meat samples were heated in a water bath until the inner temperature reached 72–76°C.

Figure 3. Samples of meat taken by hollow drill.

The texture analyzer TMS PRO with a 1000 N force transducer and blade movement speed of 500 mm min^{-1} was used for the WB method. Total shear energy consumption during cutting the probe was estimated by calculating the surface area under the force dynamic curve (Fig. 4). The blades in both (WB and GIM) devices had similar configurations with a 60° V-shaped incision and thickness of 1.016 mm).

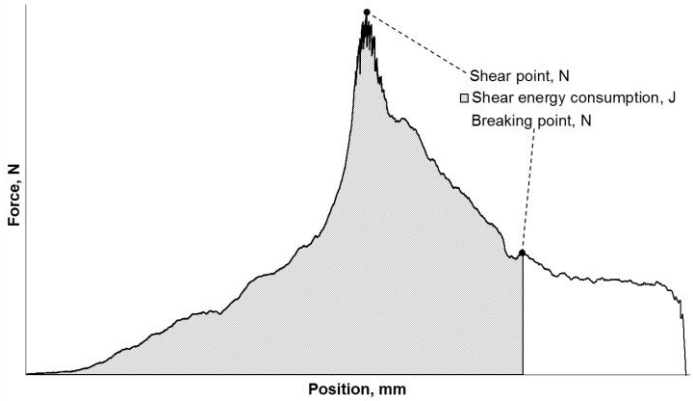

Figure 4. Force dynamics during the Warner-Bratzler test and determination of shear energy by calculating the surface area under the curve (Luno et al., 1999).

To assess the effect of ageing on the shear energy of samples, a one-way analysis of variance was performed with the spreadsheet program Excel 2010. For determination of statistical difference between the devices, student's t-test was used. The mean differences in shear energy between ageing days were evaluated by the statistical package R (R Core Team, 2013).

RESULTS AND DISCUSSION

The data of the WB method showed lower shear energy consumption when cutting raw meat samples compared to thermally treated ones ($P < 0.001$). The latter consumed 0.62 J more energy in comparison with raw meat ($P = 0.001$) (Fig. 5). The parameters of raw and thermally treated meat samples did not differ significantly ($P = 0.880$) when using the GIM method. It can be concluded that the GIM method is not suitable for determining the differences between boiled and raw meat.

Estimation of shear energy is especially important in the course of aging, because a decrease in it characterizes the enzymatic processes in the muscle, which have an effect on the muscle fibres where myofibrillar proteins degrade and meat becomes softer (Koohmaraie et al., 1995). The shear energy consumption measured by the WB method in our experiments decreased significantly ($P < 0.001$) during ageing (Fig. 6a). The shear energy of thermally treated meat (compared to raw meat) was 0.8 J higher at the beginning of ageing (day 2), however, at the end of ageing (day 35), the difference had decreased to 0.35 J.

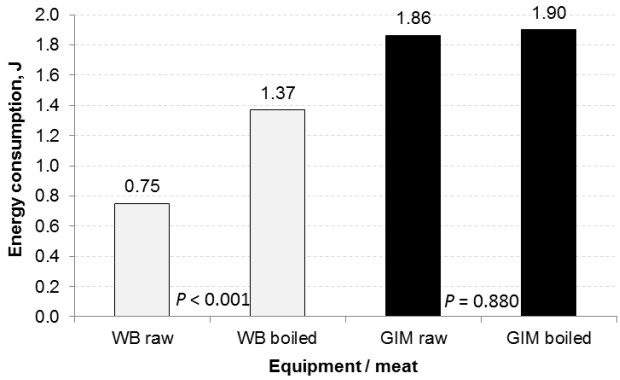

Figure 5. The shear energy consumption of raw and thermally treated meat in comparison with the WB and the GIM methods.

The shear energy consumed by the GIM method for cutting raw meat samples decreased even more during ageing (Fig. 6b), with a little bit lower statistical reliability ($P = 0.028$).

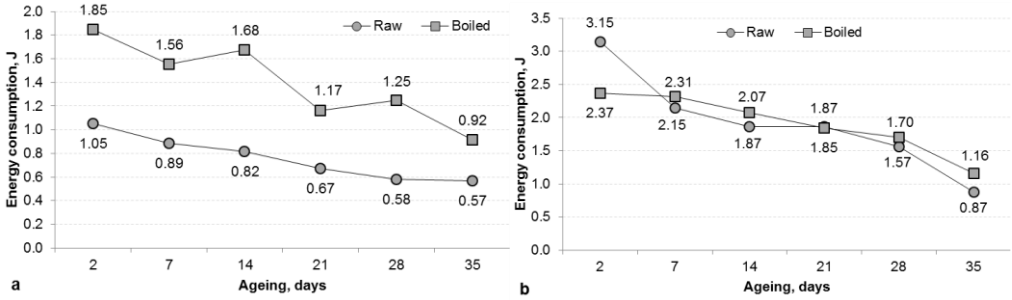

Figure 6. Changes in shear energy consumption by raw and thermally treated meat during ageing estimated with WB (a) and GIM (b) devices.

Different studies about the effect of beef ageing have produced somewhat different results. Roncalés et al. (1995) found that increasing MLD muscle ageing time changed the texture of the meat – meat tenderised throughout the whole ageing period. Huff & Parrish (1993) and Sañudos et al. (2004) concluded that 21-day ageing of MLD muscles decreased the shear force evenly in time. Our experimental data confirmed this statement. Vieira et al. (2007) and Muchenje et al. (2008) obtained different results – the conclusion of the first study was that the WB shear force value of MLD muscle decreased ($P < 0.1$) between 14 and 28 ageing days, and the second study showed that MLD muscle already reached the final tenderness by the second day of ageing and the WB shear force did not reduce significantly later (up to the 21st ageing day).

Accioli et al. (1995) found that the WB shear force values differed significantly between the 9th and 16th days of ageing, however, there were no significant differences between the 16th and 23rd day.

In our investigation, the average shear energy of raw and thermally treated meat samples decreased during ageing by using both (WB and GIM) meat shearing methods (Tables 1, 2, Figs 7, 8).

Table 1. The effect of ageing on raw meat shear energy consumption by using the WB and GIM methods (a, b, c, and d – the means within each effect with one letter in common do not differ significantly, $P > 0.05$)

Energy consumption, J	Ageing, days					
	2	7	14	21	28	35
WB method	1.05^{a}	0.89^{ab}	0.82^{abc}	0.68^{bc}	0.58^{c}	0.57^{c}
GIM method	3.15^{a}	2.15^{ab}	1.87^{ab}	1.87^{ab}	1.56^{ab}	0.87^{b}

Table 2. The effect of ageing on boiled meat shear energy consumption by using the WB and GIM methods (a, b, c, and d – the means within each effect with one letter in common do not differ significantly, $P > 0.05$)

Energy consumption, J	Ageing, days					
	2	7	14	21	28	35
WB method	1.85^{a}	1.56^{ab}	1.68^{ab}	1.17^{bc}	1.25^{bc}	0.92^{c}
GIM method	2.37^{a}	2.31^{a}	2.07^{a}	1.85^{a}	1.71^{a}	1.16^{a}

The effect of ageing on the shear energy of raw meat is illustrated on Fig. 8. The statistical significance of this effect was < 0.001 in the case of the WB method and 0.02 in the case of the GIM method. The shear energy values obtained by the GIM method were larger compared to the WB method throughout the total ageing period. Shearing energy decreased noticeably faster at the beginning of the ageing period in comparison with the end of the period. On the second day of ageing, the shear energy consumption by the GIM was 2.1 J (3.15–1.05 J) higher than that of the WB. At the end of the ageing period (35 days), this difference had decreased to 0.3 J. These data indicate the much greater sensitivity of the GIM method in the first week of ageing, compared to the WB method. Therefore, development of a new express device for evaluation of the initial stage of meat ageing would be reasonable.

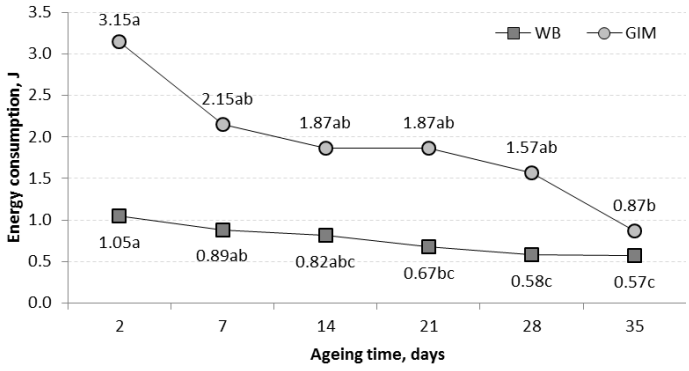

Figure 7. Changes in raw meat shear energy consumption measured by WB and GIM devices during the beef ageing period.

Thermally treated and raw meat showed similar trends in the changes of shear energy consumption for both (WB and GIS) methods (Table 2, Fig. 8) with differences in statistical significance. Shearing of thermally treated meat with the WB method showed a significant decrease in shear energy consumption during ageing days ($P < 0.001$), but the GIM method did not show a significant difference ($P = 0.38$).

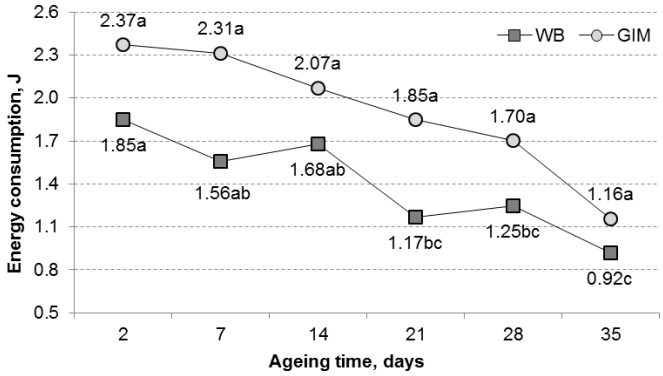

Figure 8. Changes in thermally treated meat shear energy consumption measured by WB and GIM devices during the ageing period.

CONCLUSIONS

1. The effect of ageing on the structure of meat can be determined both by the classical (WB) and the gravitational impulse method (GIM).

2. The GIM method is more sensitive in determining the tenderness of aged raw meat compared to the WB method. The advantage of the gravitational method is its simplicity.

3. On the base of the GIM method, development of a new texture analyzer for meat industry would be reasonable.

REFERENCES

Accioli, A., Franci, O., Sargentini, C., Pugliese, C., Bozzi, R. & Lucifero, M. 1995. Effetto della frollatura sulle caratteristiche della carni di vitelloni Chianini da 16 a 24 mesi di eta. Atti XI Congresso Nazionale ASPA-Grado, 359–360.

Field, R.A., Riley, M.C. & Chang, Y.O. 1971. Free amino acid changes in different aged bovine muscles and their relationship to shear values. *Journal of Food Science* **36**, 611–612.

Hanzelková, Š., Simeonovová, J., Hampel, D., Dufek, A. & Šubrt, J. 2011. The effect of breed, sex and ageing time on tenderness of beef meat. *Acta Vet.* Brno, **80**, 191–196.

Hopkins, D.L., Toohey, E.S., Kerr, M.J. & van de Ven, R. 2011. Comparison of two instruments (G2 Tenderometerand a Lloyd Texture analyser) for measuring the shear force of cooked meat. *Animal Prod. Sci.* **51**, 71–76.

Huff, E.J. & Parrish, F.C.Jr. 1993. Bovine longissimus muscle tenderness as affected by post-mortem ageing time, animal age and sex. *J. Food Sci.* **58**, 713–716.

Jennings, T.G., Berry, B.W. & Joseph, A.L. 1978. Influence of fat thickness, marbling and length of ageing on beef palatability and shelf-life characteristics. *J. Animal Sci.* **46**, 658–665.

Koohmaraie, M., Wheeler, T.L. & Shackelford, S.D. 1995. Beef tenderness: regulation and prediction. USDA-ARS U. S. Meat Animal Research Center **9**, 1–25.

Luno, M., Beltran, J.A., Jaime, I. & Roncales, P. 1999. Textural assessment of clenbuterol treatment in beef. *Meat Sci.* **51**, 297–303.

Mottram, D.S. 1998. Flavour formation in meat and meat products: A review. *Food Chem.* **62**, 415–424.

Muchenje, V., Dzama, K., Chimonyo, M., Raats, J.G. & Strydom, P.E. 2008. Meat quality of Nguni, Bonsmara and Aberdeen Angus steers raised on natural pasture in the Eastern Cape, South Africa. *Meat Sci.* **79**, 20–28.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Roncalés, P., Geesink, G.H., Van Laack, R.L.J.M., Jaime, I., Beltrán, J.A., Barnier, V.M.H. & Smulders, F.J.M. 1995. Meat tenderisation: Enzymatic mechanisms. Source: Ouali, A, Demeyer, D.I., Smulders, J.M. (eds), *Expression of tissue proteinases and regulation of protein degradationas related to meat quality*. ECCEAMST, Utrecht, 311–332.

Sañudo, C., Macie, E.S., Olleta, J.L., Villarroel, M., Panea, B. & Albertí, P. 2004. The effects of slaughter weight, breed type and ageing time on beef meat quality using two different texture devices. *Meat Sci.* **66**, 925–932.

Savell, J., Miller, R., Wheeler, T., Koohmaraie, M., Shackelford, S., Morgan, B., Calkins, C., Miller, M., Dikeman, M., McKeith, F., Dolezal, G., Henning, B., Busboom, J., West, R., Parrish, F. & Williams, S. 2013. Standardized Warner-Bratzler Shear Force Procedure for Genetic Evaluation. http://meat.tamu.edu/research/shear-force-standards/ Last visited 03.07.2013.

Wheeler, T.L., Shackelford, S.D., Johnson, L.P., Miller, M.F. & Koohmaraie, M. 1997. A Comparison of Warner-Bratzler Shear Force Assessment Within and Among Institutions. *J. Animal Sci.* **75**, 2423–2432.

Wheeler, T.L., Shackelford, S.D. & Koohmaraie, M. 2013. Warner-Bratzler Shear Force Protocol. USDA- ARS U.S. Meat Animal Research Center. http://www.ars.usda.gov/SP2UserFiles/Place/54380530/protocols/Warner-BratzlerShearForceProtocol.pdf/

Vieira, C., Cerdeño, A., Serrano, E., Lavín, P. & Mantecón, A. R. 2007. Breed and ageing extent on carcass and meat quality of beef from adult steers (oxen). *Livestock Sci.* **107**, 62–69.

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Estimation of Particle Size Distribution in Bovine Colostrum Whey by Dynamic Light Scattering (DLS) Method

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Abstract. Colostrum whey consist bioactive compounds in considerable concentration. For isolation of these compounds the particle size has crucial importance. The aim of this work was to study possibilities of using dynamic light scattering method – DLS (Malvern Zetasizer Nano ZS) for colostrum whey particle size distribution estimation. The first and second milking colostrum samples were skimmed by centrifugal separation and casein of these was enzymatically coagulated by rennet (chymosin). Obtained whey was diluted (1:200) by distilled water and filtered (cut-off 0.45 μm) to get probes for estimation of particle size. Particle size distribution in colostrum whey probes had maximally three peaks and polydispersity indices from 0.157 to 0.541. Prevailing peak of the distribution was found at size from 144 to 210 nm, which apparently corresponds to hydrodynamic diameter of immunoglobulin IgG1.

Key words: Milk, Colostrum, Whey, Particle size distribution, immunoglobulin.

INTRODUCTION

Colostrum is a complex fluid rich in nutrients and is also characterised by its high level of bioactive (e.g. antimicrobial) components, like immunoglobulins (Ig), especially IgG1, growth factors, especially insulin-like growth factor-1 (IGF-1), transforming growth factor beta-2 (TGF-b2) and growth hormone (GH) as well as lactoferrin, lysozyme and lactoperoxidase (table 1). Colostrum whey contains a significant amount of those bioactive compounds (Pakkanen & Aalto, 1997; Elfstrand et al., 2002; Marnila & Korhonen, 2002; Kehoe et al., 2007), which can be used in livestock husbandry, in food and feed supplements, in medical products, etc. Concentration of proteins and/or bioactive components in bovine colostrum and milk are shown in Table 1.

The pharmaceutical and biotechnological industries have recently shown interest in bovine colostrum as a source of growth factors and other specific bioactive components. Also, a multitude of health products and foods made from various colostrum fractions have been launched on the market. Fractionation of colostrum components (immunoglobulins, lactoferrin, glycomacropeptides, etc.) from colostral whey may result in profitable returns and hence, more research into effects of fractions or individual components compared to whole colostrum is being undertaken. (Tripathi & Vashishtha, 2006)

Table 1. Concentration of proteins and/or bioactive components in bovine colostrum and milk. Data adapted from: Pakkanen & Aalto 1997; Marnila & Korhonen, 2002; Elfstrand et al., 2002; Kehoe et al., 2007

Protein and Growth Factors	Colostrum	Milk
Casein	26 g l^{-1}	29 g l^{-1}
α-lactalbumin	2 g l^{-1}	1.4 g l^{-1}
β-lactoglobulin	8 g l^{-1}	3.3 g l^{-1}
IgG1	48 k–87 g l^{-1}	0.7 g l^{-1}
IgG2	1.6–2.9 g l^{-1}	0.05 g l^{-1}
IgM	3.7–6.1 g l^{-1}	0.05 g l^{-1}
IgA	3.2–6.2 g l^{-1}	0.1 g l^{-1}
Serum Albumin	1.2 g l^{-1}	0.4 g l^{-1}
Lactoferrin	1.0–2.0 g l^{-1}	0.1 g l^{-1}
Lactoperoxydase	30 mg l^{-1}	20 mg l^{-1}
TGF-b2	20–40 mg l^{-1}	1–2 μg l^{-1}
IGF-1	0.1–2 mg l^{-1}	25 μg l^{-1}
Lysozyme	0.1–0.7 mg l^{-1}	0.1–0.3 mg l^{-1}

Values are depending on postpartum timeline and cow´s individuality. Data represent range.

To extract bioactive compounds from colostrum membrane filtration may be used (Elfstrand et al., 2002; Venkiteshwaran et al., 2008). Based on the particle size distribution it is possible to select optimum separation technologies and also evaluate the protein composition of colostrum whey. Although there are some references concerning skimmed milk (Beliciu & Moraru, 2009) and whey (Giroux et al., 2009) particle size distribution (PSD) by dynamic light scattering (DLS) method, there is no such data about PSD in bovine colostrum available. An important factor in use of separation technologies and of DLS is the knowledge about possible polydispersity which is caused by considerable differences in particle size. In application of DLS method for investigation of particle size it is essential to prevent:

1. the presence of large particles in the sample
2. excessively high concentration of nanoparticles in the sample
3. denaturation or aggregation of particles due to pH or some other co-factor as temperature, concentration etc. (Dalgleish & Hallett, 1995; Alexander & Dalgleish, 2006; Beliciu & Moraru, 2009).

The aim of current research was to evaluate the suitability of DLS method for the estimation of particle size distribution and its polydispersity in colostrum whey.

MATERIALS AND METHODS

First and second milking colostrum (1.5 l) was collected and frozen at the EULS Märja experimental cowshed. Five first and seven second milking samples of colostrum were under investigation. To ensure efficient separation of fat frozen colostrum was warmed up to separation temperature 55°C in water bath. Fat separation process was conducted by separator Armfield FT15 (10,000 rpm). Casein was precipitated at 35°C using rennet (Formatase 2200 TL) which is derived from a fermentation process of the fungus *Rhizomucor miehei* and does not affect whey proteins. In order to secure large particles (casein-dust, fat, etc.) free and transparent solution, colostrum whey was diluted (1 : 200) and filtered by syringe filter (cut-off

0.45 μm). Since whey proteins are more stable close to neutral pH, distilled water (pH 6.8–7.2) was used as diluting solution for reduction of particles concentration in current research. Isoelectric points of whey proteins are presented in Table 2 (Pouliot & Gauthier, 2006). Dilution of whey was carried out before filtering to prevent clogging of the filter. The effect of pH and dilution environment on colostrum whey particle size distribution was not studied because this needs a detailed investigation.

Table 2. Isoelectric point of whey proteins and growth factors present in milk (Pouliot & Gauthier, 2006)

Milk protein	IgG1	IgG2	BSA	βLg	αLa	GF	LF	LP
Isoelectrical point, pH	6.5	8	4.7	5	4.7	4.7–9.5	9	10

BSA – serumalbumin, βLg – β-lactoglobulin, αLa – α-lactalbumin, GF – Growth factors, LF – Lactoferrin, LP – Lactoperoxydase.

For estimation of colostrum whey particle size distribution Malvern Zetasizer Nano-ZS analyser which is based on dynamic light scattering (DLS) method was used. This device measures the time dependent fluctuations in the scattering intensity of light to determine the translational diffusion coefficient, and subsequently the hydrodynamic diameter by the Stokes-Einstein equation. Each measurement consisted of 3 subsequent individual runs of which the average result was calculated. Measurements were conducted at 22.0ºC and the light scattering was detected at 173 degrees. The detection range of device is from 0.1 nm to 10 μm. The data obtained by the Malvern Zetasizer Nano-ZS analyser was exported into Microsoft Excel for further analyses.

RESULTS AND DISCUSSION

The particles size distribution (PSD) of diluted and filtered colostrum whey (DFCW) was found to be in wide range and can therefore be described as multimodal dispersion (indicates polydispersity). Polydispersity in PSD is reflected clearly by existence of three peaks (Fig. 1).

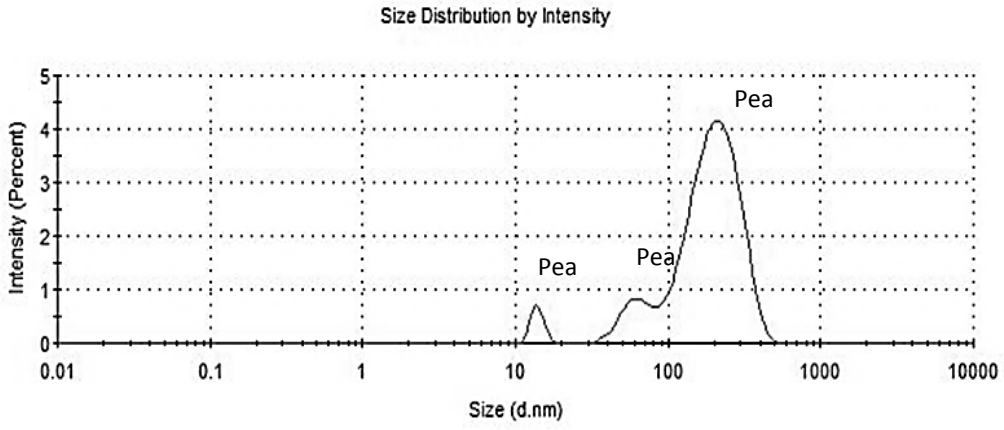

Figure 1. Example of particles size distribution (PSD) graph with three peaks of diluted and filtered first milking colostrum whey (cow No. 550).

PSD is usually described by overall average or cumulant average (z-average) of particle diameter (size). In case of polydisperse solutions interpretation of PSD results using z-average might be insufficient. To describe the polydispersity of the PSD in DFCW z-average, mean intensity size and area intensity percentage of each peak and polydispersity index (PDI) were calculated (Table 3).

Table 3. Results of particle size distribution in colostrum whey estimated by DSL method. (PDI – polydispersity index, MI – mean intensity which corresponds to mode of the peak, AI – area intensity which corresponds to partial area under the peak of the total distribution)

Cow No/ milking	z-average, nm	PDI	Peak 1 MI, nm	Peak 2 MI, nm	Peak 3 MI, nm	Peak 1 AI, %	Peak 1 AI, %	Peak 1 AI, %
528/ I	94.5	0.541	177.7	45.1	11.6	80.1	9.1	7.5
624/ I	106.5	0.533	209.6	51.8	12.6	80.3	13.5	6.3
550/ I	122.8	0.458	211.0	61.1	14.0	96.1	3.9	0.0
70/ I	132.2	0.226	167.7	30.9	0.0	99.2	0.8	0.0
439/ I	113.5	0.310	173.9	39.8	16.7	92.8	3.7	3.5
Average of 1st milking	113.9	0.414	188.0	45.7	11.0	89.7	6.20	3.46
St.dev.	13.0	0.13	18.5	10.3	5.75	8.0	4.53	3.11
550/ II	148.3	0.290	200.5	63.4	0.0	97.5	2.5	0.0
92/ II	132.3	0.192	155.6	0.0	0.0	100.0	0.0	0.0
44/ II	105.1	0.311	144.1	13.7	0.0	96.1	3,9	0.0
60/ II	185.5	0.157	199.8	0.0	0.0	100.0	0.0	0.0
17/ II	129.7	0.239	168.9	0.0	0.0	100.0	0.0	0.0
70/ II	138.0	0.271	184.1	50.48	0.0	97.4	2.6	0.0
76/ II	135.0	0.328	196.9	43.86	0.0	90.1	9.9	0.0
Average of 2nd milking	139.1	0.255	178.6	24.50	0.0	97.3	2.7	0.0
St.dev.	22.51	0.06	21.07	25.29	0.0	3.28	3.28	0.0
Average of 1st and 2nd milking	126.5	0.335	183.3	35.1	5.50	93.5	4.5	1.7
St.dev.	22.82	0.12	20.57	22.95	6.56	6.86	4.22	2.63

PSD of first milking probes had 2–3 peaks with average mean intensity sizes 188.0, 45.7 and 11.0 nm for peaks 1, 2 and 3 (may also be absent) respectively. Mean intensity and area intensity varied between 173.9–209.6 nm and 80.1–99.2% for peaks 1 and between 30.9–61.1 nm and 0.8–13.5% for peak 2. PSD of second milking colostrum probes had 1–2 peaks with average mean intensity sizes of 178.6 nm for peak 1 and 24.5 nm for peak 2. Mean intensity and area intensity of the dominating peak 1 varied between 144.1–200.5 nm and 90.1–100.0% respectively.

Polydispersity index (PDI) indicates how homogenous the probes appeared to be, at least from a light scattering perspective. PDI larger than 0.2 indicates that the simple cumulant fitting is not a complete representation and that more than a single species are present (Nobbmann, 2007). It is acknowledged that polydispersity affects the DLS measurement results. Although it is also clear that the mean intensity results of the dominating peak are less affected and the role of smallest particles to it may be neglected. The second milking colostrum probes showed clearly more homogenous PSD results having only two peaks maximally and lower mean PDI (0.255) compared

to first milking colostrum probes (mean PDI = 0.414). This can be explained by rapid postpartum changes in colostrum composition (Elfstrand et al., 2002). According to published investigations about the content of colostrum and normal milk protein compounds (Table 1), and the fact that casein and fat fractions were removed from probes, it can be assumed that dominating peak of PSD in our experiments represented major colloidal compound of colostrum whey – immunoglobulin IgG1.

Because of wide variance in polydispesity index values (PDI = 0.157–0.541) and considerably large mean size of dominating particles certain aggregation of those in DFCW probes can be assumed also. There are several studies about the influence of various factors including storage conditions and processing variables such as heat, pH and pressure on stability of bovine IgG (Elfstrand et al., 2002; Godden et al., 2006; Mcmartin et al., 2006; Indyk et al., 2007). Still, from the point of view of PSD estimation by DLS-method the question about possible aggregation of IgG remains to be answered.

Further on more profound investigations about sample treatment impact (dilution media, pH, heat treatment, refrigerated preservation, etc.) towards PSD of bovine colostrum whey proteins are required. The certain aggregation degree of whey proteins (especially IgG1) by regulation of temperature and pH could even enhance efficiency of filtration or other extraction technology (bigger particles ensure more effective separation). However, it is essential to preserve the bioactivity of protein during this kind of treatment.

CONCLUSIONS

This study showed that DLS method is applicable for evaluation of particle size distribution and polydispersity of the colostrum whey proteins. Based on the cited knowledge about content of colostrum protein compounds and the fact that casein and fat fractions were removed, it can be assumed that highest mean intensity of PSD in our study represents major colloidal compound of colostrum whey – immunoglobulin IgG1. Further research about the effect of pH and dilution environment in the phase of sample preparation and possible aggregation of IgG on colostrum whey particle size estimation by DSL-method is needed.

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REFERENCES

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

Beliciu, C.M., & Moraru, C.I. 2009. Effect of solvent and temperature on the size distribution of casein micelles measured by dynamic light scattering. *J. Dairy Sci.* **92**, 1829–1839.

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

Elfstrand, L., Lindmark-Mansson, H., Paulsson, M., Nyberg, L. & Akesson, B. 2002. Immunoglobulins, growth factors and growth hormone in bovine colostrum and the effects of processing. *Int. Dairy J*. **12**, 879–887.

Giroux, H.J., Houde, J. & Britten, M. 2010. Preparation of nanoparticles from denatured whey protein by pH-cycling treatment. *Food Hydrocolloids*. **24**(4), 341–346

Godden, S., McMartin, S., Feirtag, J., Stabel, J., Bey, R., Goyal, S., Metzger, L., Fetrow, J., Wells, S. & Chester-Jones, H. 2006. Heat-Treatment of bovine colostrum. II: Effects of heating duration on pathogen viability and immunoglobulin G. *J. Dairy Sci.* **89**, 3476–3483.

Indyk, H.E., Williams, J.W. & Patel, H.A. 2007. Analysis of denaturation of bovine IgG by heat and high pressure using an optical biosensor. *Int. Dairy J.* **18**, 359–366.

Kehoe, S.I., Jayarao, B.M. & Heinrichs, A.J. 2007. A survey of bovine colostrum composition and colostrum management practices on Pennsylvania dairy farms. *J. Dairy Sci.* **90**, 4108–4116.

Marnila, P. & Korhonen, H. 2002. Colostrum. *Encyclopedia of Dairy Sciences.* **1**, 471–477.

McMartin, S., Godden, S., Metzger, L., Feirtag, J., Bey, R., Stabel, J., Goyal, S., Fetrow, J., Wells, S. & Chester-Jones, H. 2006. Heat treatment of bovine colostrum. I: Effects of temperature on viscosity and immunoglobulin G level. *J. Dairy Sci.* **89**, 2110–2118

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

Pakkanen, R. & Aalto, J. 1997. Growth Factors and Antimicrobical Factors of Bovine Colostrum. *Int. Dairy J.* **7**, 285–297.

Pouliot, Y. & Gauthier, S.F. 2006. Review. Milk growth factors as health products: Some technological aspects. *Int. Dairy J.* **16**, 1415–1420.

Tripathi, V. & Vashishtha, B. 2006. Bioactive Compounds of Colostrum and Its Application. *Food Reviews International*. **22**, 225–244.

Venkiteshwaran, A., Heider, P., Teysseyre, L. & Belfort, G. 2008. Selective precipitation-assisted recovery of immunoglobulins from bovine serum using controlled-fouling crossflow membrane microfiltration. *Biotechnol. Bioeng.* **101**(5), 957–966.

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Use of *Delvotest T* for Quantitative Estimation of β-lactam Antibiotic Residues in Waste Milk and for Evaluation of Thermal Treatment Efficiency – a Methodical Pilot Study

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Abstract. The aim of this work was to study possibilities of using microbiological broad-spectrum inhibitor test (*Delvotest T*) for express estimation of antibiotic residues in bovine milk. For quantitative estimation the waste-milk samples were stepwise diluted (dilution array) by antibiotic-free milk until negative test result was achieved. Another objective was to evaluate heat treatment efficiency of waste-milk in order to degrade antibiotic residues in it. Heat treatment (in water bath) at 90°C was chosen according to references in literature. In order to study certain drug residue the waste-milk samples were collected at the next milking after medical treatment. Two different drugs were investigated: Carepen and Norocillin. Preliminary results indicated that the average concentration of antibiotic residue (active substance of drugs) exceeded European MRL (Maximum Residue Limit) 4,100 (± 2,408) and 13.0 (± 5.7) times respectively. The average heat treatment duration at 90°C (until negative test result of *Delvotest T*) was 15.0 (± 5.0) and 7.8 (± 1.5) hours respectively.

Key words: Waste-milk, Antibiotic residues, *Delvotest T*, Heat treatment.

INTRODUCTION

It is widely known that improper use of antibiotics may lead to residues in milk, especially when withdrawal times are not respected. These residues can be dangerous for human health. They may cause allergic reactions, antibiotic resistance of pathogens etc. Antibiotic residues can also create a technological problem for industry production concerning bacterial fermentation processes in dairy products (Packham et al., 2001).

According to Statistics Estonia (www.stat.ee, 2014), the production of raw milk in 2012 was 721,200 tons. In addition to that, there is an estimated 13–19 thousand tons of raw milk containing antibiotic residue (AR). This milk is also known as waste-milk (WM), which is still an unused resource. For the use of that resource inactivation or degradation of AR is to be carried out. To control these processes estimation of antibiotic concentration in milk is of crucial importance.

Nowadays there are several receptor-based lateral flow assay tests employed routinely at the farm level and in the dairy industry because they are fast and simple to use. Microbiological test kits based on the inhibition of *Geobacillus stearothermophilus* are most frequently used for the screening analysis of milk in farms and dairy industries. According to Reybroeck and Ooghe (2012) the *Delvotest T* is a

new version of *Delvotest SP-NT* with an improved detection capability. It detects β-lactam (and many others) antibiotics efficiently in respect of actual Maximum Residue Limit (MRL) legislation (Commission Regulation (EU) No. 37/2010 and amendments). The important advantage of microbiological methods is that false negative samples can be almost excluded. Although it must be acknowledged that false positive results could occur if other than antibiotic inhibitors are present. Disadvantage of this method is that threshold level (MRL) of antibiotic in milk could be estimated only. For concentration studies expensive and sophisticated methods basing on chromatography should be used.

Thermal treatment is likely one possibility for antibiotics degradation in milk. There are several studies concerning thermal degradation of β-lactam antibiotics from a pharmaceutical aspect in aqueous solutions under different storage conditions, at different pH, etc. Also there are some studies about the effects of temperature on β-lactam antibiotics in foodstuffs (Moats, 1999; Hanway et al., 2005; Zorraquino et al., 2008; Roca et al., 2010, 2011, 2012).

Roca et al. (2011) investigated the effect of heating on the stability of β-lactams (penicillins and cephalosporins) in skim milk (skim milk powder was reconstituted to 10%). The results indicated that the conventional milk processing techniques causes minor losses in the concentration of these antibiotics and therefore do not prevent these antimicrobial substances from reaching consumers. Although it was clearly shown that milk sterilization at 120°C for 20 min had significant impact towards stability of β-lactams. Zorraquino et al. (2008) used a bioassay based method on the inhibition of *Geobacillus stearothermophilus* var. *calidolactis.* They studied three industrial heat treatments regimes. Of those classic sterilization (120°C for 20 min) showed convincingly highest level of heat inactivation of over 65% for penicillins although penicillin G concentrations in fortified milk samples were used only three times over MRL. However it can be concluded that duration of thermal treatment is a substantial factor concerning inactivation of β-lactam antibiotics.

Researches dealing with quantity of antibiotic residue in milk have been conducted mostly by spiking/fortifying milk (skim milk or commercial UHT milk) with antibiotic and the analysis of drug concentration performed by sophisticated laboratory equipment such as liquid chromatography etc. The aim of this work was to study actually treated cow's raw milk and to conduct a pilot experiment for investigation of a simple alternative for estimation of the quantity of (β-lactam) antibiotic residues in milk by *Delvotest T*. Additional goal was to scan thermal degradation of such milk.

MATERIALS AND METHODS

The samples of raw milk for this study were collected from treated cow´s in Põlva and Hummuli farms and cooled down. Two veterinary drugs Carepen and Norocillin were under investigation in current study. Both drugs contained the same active substance (PBP – procaine benzylpenicillin) but concentrations and route of administration were different (Table 1). Carepen was used intramammary in case of mastitis and Norocillin intramusculary in case of leg disease. The samples (1.5 l per cow) were collected during next milking after the treatment (assuming that the milk

contains maximal possible concentration of antibiotic residue then). Experiments were conducted on the following day after collection of the samples.

Table 1. Description of drugs studied in current work, EU maximum residue limit (MRL) and detection limit (DL) of *Delvotest T*

Drug	AS*	Route of administration	Drug admin. (ml)	AS in drug (mg ml^{-1})	AS admin. (ml)	MRL (μg kg^{-1})	DL of Delvotest T (μg kg^{-1})
Carepen	PBP**	Intramammary	10	60	600	4	4
Norocillin	PBP**	Intramusculary	40	300	1,200	4	4

*AS is active substance

**PBP is procaine benzylpenicillin

Quantitative estimation of antibiotic by *Delvotest T*

The *Delvotest T* is a microbiological broadspectrum inhibitor test. To screen milk on the presence of antimicrobials it uses *Geobacillus stearothermophilus* var. *calidolactis* as a test organism. Microplates were incubated (floating on the water surface) in a waterbath at 64°C. After 3 h incubation, the color change of the pH indicator in the agar of the wells (yellow–negative, purple–positive) was recorded. For quantitative AR (antibiotic residue) estimation the waste-milk was diluted by antibiotic-free milk in order to achieve an array of different concentrations (dilution factors) of WM (Fig. 1 a). The 2.5% fat content commercial milk used as dilution environment was purchased from ordinary public store and tested by *Delvotest T* (no positive test results were found in current study).

Table 2. Example of dilution arrays used for quantitive estimation of antibiotic residue (AR). Bold represents AR below MRL (Maximum Residue Limit)

Veterinary Drug	Carepen					
1st dilution array	500	1,000	**3,000**	5,000	7,000	9,000
Delvotest T results	pos	pos	**neg**	neg	neg	neg
2nd dilution array to refine results	1,000*	**1,500**	2,000	2,500	3,000*	
Delvotest T results	pos	**neg**	neg	neg	neg	
Veterinary Drug	Norocillin					
1st dilution array	10	**30**	50	100	150	200
Delvotest T results	pos	**neg**	neg	neg	neg	neg
2nd dilution array to refine results	10*	**15**	20	25	30*	
Delvotest T results	pos	**neg**	neg	neg	neg	

* – dilution was made as control to ensure reliability of results

The dilution factor at which negative test result occurred in 1st dilution array was refined by 2nd dilution array and considered as detection limit (DL) of *Delvotest T* witch conveniently equals to MRL. Example of dilution arrays for quantitive estimation of antibiotic residue (AR) in case of Norocillin and Carepen are shown in Table 2. Negative results were taken into account only in case of clear colour change.

Heat treatment

For the heat treatment 50 ml sealed sample container was placed in thermostatic bath at 90°C. The 100 µl probes for detection of antibiotic residue (AR) were taken from the container hourly. Heat treatment was considered sufficient, and degradation of AR completed at a probe with negative test result (Fig. 1b). Objective was to establish the thermal degradation duration (TDD) at which negative test occurred (AR < MRL).

Roca et al (2011) found that the half-life of benzylpenicillin (PBP) at 90°C is 52 min. In current study this value was taken for bases and the predicted duration of thermal degradation (Table 3) was calculated by equations 1, 2, where PT is predicted time, $t^{1/2}$ is half-life of benzylpenicillin at 90°C (according to Roca et al (2011), 52 min), $n^{1/2}$ is count of $t^{1/2}$ (half-life of PBP), AR is estimated antibiotic residue and MRL is maximum residue limit of AR.

$$PT = n^{1/2} t^{1/2} \quad (1)$$

$$n^{1/2} = (\log AR - \log MRL)/\log 2 \quad (2)$$

Figure 1. *Delvotest T* results: a) estimation of concentration (dilution factors: 500–2,000) b) influence of thermal treatment duration (4–11 hours). Arrow points to the lowest dilution factor (1,500) and thermal treatment duration (10 hours), at which negative test occurred (AR < MRL). Originally colour photos are represented here in grayscale mode.

RESULTS AND DISCUSSION

Table 3 presents estimated quantity of antibiotic residue (AR) in raw waste-milk and thermal degradation time of veterinary drugs (Carepen and Norocillin) AR. The difference between average AR concentrations of two drugs was remarkable (315 times). Significantly higher AR concentrations of Carepen can be explained by route of administration (intramammary or intramusculary). In addition to that the composition of drug (excipient) might have some impact to the results. Suggested 1st dilution arrays for quantity estimation of Norocillin and Carepen AR are presented in Table 2.

High standard deviation values concerning negative *Delvotest T* results (DF neg in Table 3), computational concentrations of AR (CC of AR) and thermal degradation duration (TDD) can be explained by differences in waste-milk quantity (WMQ) of particular milking. Negative correlation between AR concentration and WMQ also confirms that. High standard deviation value of TDD compared to PT of TDD can be

explained by cow´s individuality (milk production level, composition differences, etc.) which influences quantity of AR.

Initially just slight difference (in view of AR concentration) concerning the duration of thermal degradation of different WM samples as well as different drugs can be simply explained by exponential character of half-life degradation.

Remarkable correlation between computational concentration of AR and thermal degradation time supports the assumption that quantitative estimation of AR could be carried out by using *Delvotest T* on an array of diluted milk samples.

Table 3. Results of quantitative estimation and duration of thermal degradation of antibiotic residue in waste-milk

Cow No	Drug	DF neg	CC of AR, μg kg^{-1}	TDD till neg, h	PT of TDD*, h	WMQ, kg
4,859	Carepen	1,500	6,000	10	9.2	16
5,364	Carepen	7,500	30,000	23	11.2	13
5,894	Carepen	3,500	14,000	19	10.8	12
6,805	Carepen	5,500	22,000	15	11.3	11
6,737	Carepen	2,500	10,000	14	9.8	12
Average		4,100	16,400	15.0	10.24	12.8
St dev		2,408	9,633	5.0	0.79	1.92
Concentration correlation				0.82	0.84	-0.45
439	Norocillin	5	20	6	2.0	13
123	Norocillin	15	60	7	3.4	6
6,790	Norocillin	20	80	10	3.8	9
5,979	Norocillin	15	60	8	3.4	10
5,286	Norocillin	10	40	8	2.9	16
Average		13.0	52.0	7.8	3.1	10.8
St dev		5.70	22.80	1.48	0.69	3.83
Concentration correlation				0.83	0.80	-0.65

* – Based on the half-life of benzylpenicillin at 90°C (Roca et al., 2011)
DF neg – Dilution factors of negative *Delvotest T* results
CC of AR – Computational concentration (CC) of AR based on DL=MRL (4 μg kg^{-1})
TDD till neg – Thermal treatment (90°C) duration till neg. test result occurred
PT of TDD – Predicted time of thermal degradation duration (TDD)
WMQ – waste-milk quantity

Table 3 also presents predicted time (PT) of thermal degradation duration (TDD) based on the half-life of benzylpenicillin at 90°C described by Roca`s research group and estimated AR concentration results from our study. Compared to PT of TDD, the TDD of Carepen and Norocillin AR was significantly longer. This can be explained by the fact that the results were expressed only in case of clear colour change. So considering that, the actual concentration of AR might have been lower and/or the duration of AR degradation therefore shorter. It also might be caused by the composition of drug (excipient), route of administration, cow´s individuality, etc.

Further studies are needed to work out determined procedures for implementing proposed method in practice and for evaluation of it.

CONCLUSIONS

For quantitative estimation of antibiotic residue in milk expensive and sophisticated laboratory equipment such as liquid chromatography etc. is normally used. The current pilot study demonstrates simple potential alternative for express quantitative estimation of antibiotic residues in bovine milk by applying *Delvotest T* to an array of diluted milk samples. This method is probably applicable in case of receptor-based lateral flow assay tests (testkits) also, which are employed routinely at the farm level and in the dairy industry because they are fast and simple to use. Preliminary conclusions of current study are: 1) proposed express method for quantitative estimation of AR in waste-milk and in heat treated waste-milk by *Delvotest T* is applicable; 2) the heat treatment study confirmed that procaine benzylpenicillin (PBP) in waste-milk can be degraded by long-term thermal treatment at 90°C.

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REFERENCES

European Council. 2010. Council Regulation 37/2010 of the European Communities. Pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Off. J. Eur. Union.* L**15**, 1b–72.

Hanway, W.H., Hansen, A.P., Anderson, K.L., Lyman, R.L. & Rushing, J.E. 2005. Inactivation of Penicillin G in Milk Using Hydrogen Peroxide, *J. Dairy Sci.* **88**, 466–469.

Moats, W.A. (1999). The effect of processing on veterinary residues in foods. Advances in experimental medicine and biology Volume: **459**, 233–241.

Packham, W., Broome, M.C., Limsowtin, G.K.Y. & Roginski. H. 2001. Limitations of standard antibiotic screening assays when applied to milk for cheesemaking. *Aust. J. of Dairy Technol.* **56**(1), 15–18.

Reybroeck, W. & Ooghe, S. 2012. Validation report of the Delvotest T. January 6th 2012, 1–13.

Roca, M., Castillo, M., Marti, P., Althaus, R.L. & Molina, M.P. 2010, Effect of Heating on the Stability of Quinolones in Milk *J. Agric. Food Chem.* **58**, 5427–5431.

Roca, M., Villegas, L., Kortabitarte, M.L., Althaus, R.L. & Molina, M.P. 2011. Effect of heat treatments on stability of β-lactams in milk, *J. Dairy Sci.* **94**, 1155–1164.

Roca, M., Althaus, R.L. & Molina, M.P. 2012. Thermodynamic analysis of the thermal stability of sulphonamides in milk using liquid chromatography tandem mass spectrometry detection. *Food Chem.* **136**(2), 376–383.

Statistics Estonia. *http://pub.stat.ee/px-web.2001/Database/Majandus/13Pellumajandus/06Pellumajandussaaduste_tootmine/02Loomakasvatussaaduste_tootmine/02Loomakasvatussaaduste_tootmine.asp.* Accessed 10.1.2014.

Zorraquino, M.A., Roca, M., Fernández, N., Molina, M.P. & Althaus, R.L. 2008. Heat inactivation of beta-lactam antibiotics in milk. *J. Food Prot.* **71**, 1193–1198.

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Effect of ultrasonic treatment on metabolic activity of *Propionibacterium shermanii*, cultivated in nutrient medium based on milk whey

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Abstract. Utilization of milk whey still remains one of the most serious problems of the modern world dairy industry. Because of high biological value of whey it can be used as nutrient media in techniques of direct microbiological synthesis of complex high-molecular substances, for example the B_{12} vitamin, which participate in various biocatalytic reactions in organism providing normal functioning of the brain, nervous and hematogenic systems.
The main industrial producer of B_{12} vitamin is *Propionibacterium shermanii* species, which can use lactose as main carbon source and can develop directly in milk whey. However, common ways of vitamin B_{12} microbiological production can't be applied on the food plants and there must be used alternative safe methods, such as ultrasonic treatment of the cell culture, which can carry out the direct modulation of metabolic activity of bacteria for increasing of a yield of B_{12} vitamin.
For the definition of ultrasonic processing influence on metabolism of *Propionibacterium freudenreichii ssp. shermanii* industrial strain it was cultivated in the nutrient media based on milk whey and treated by low intensity ultrasound at a frequency of 20 kHz within 10 and 20 min each 24 h. Received results allow to suggest that ultrasonic modulation of propionic bacteria metabolic activity can be used for an intensification of B_{12} vitamin biotechnological obtaining and manufacturing of fermented food products based on milk whey and enriched with B_{12} vitamin.

Key words: ultrasound, *Propionibacterium shermanii*, B12, cobalamine, milk whey.

INTRODUCTION

The one of the most important vitamins for the human health is the B_{12} vitamin which involves several kinds of specific forms of cobalt-containing compounds, known as cobalamins. The main industrial form of vitamin is cyanocobalamin due to its high stability. Cobalamines serve as cofactors for various important enzymes participating in maintenance of normal DNA synthesis, regeneration of methionine and other biocatalytic reactions providing normal functioning of the brain and nervous system, blood formation, etc. Deficiency of these compounds in diet can lead to different neurological diseases, vascular damage, thrombosis and even to serious form of anemia called Addison's pernicious anemia (Green & Miller, 2010).

Naturally they are synthesized only by microorganisms and contain only in food of an animal origin. The lack of these compounds leading to various diseases often can be observed in the diet through various gastric disorders, such as chronic gastritis that

lead to malabsorption of vitamin, or inadequate dietary intake, for example in vegetarian diet. Deficiency of B_{12} can be corrected by introduction special food products enriched with high amounts of vitamin which can provide adequate B_{12} intake even through a passive diffusion mechanism that allows assimilate only of 1–2% of vitamin containing in food. That type of food products also can be used as medical food for people who can't absorb vitamin B_{12} via the normal physiologic route. The recommended dietary allowance of B_{12} for males and females is 2.4 μg per day and higher, so such products should contain at least 240 μg kg^{-1} of vitamin.

However nowadays B_{12} vitamin is one of the most expensive vitamins and it is used mostly as medical agent for injections and due to its high cost it can't be used as simple food additive.

Vitamin B_{12} is produced exclusively by biosynthetic way. The main industrial producer of B_{12} vitamin is *Propionibacterium freudenreichii ssp. shermanii* which was recently used mainly as a ripening culture in Swiss-type cheeses. These microorganisms synthesize exclusively biologically active forms of vitamin and able to use a variety of carbon substrates, including lactose and lactic acid, that allows them to grow in milk whey (Falentin et al., 2010).

Milk whey contains from 4.2 to 7.5% of solids, most of which are disaccharide lactose (3.5–5.2%), a large number of organic and inorganic compounds containing essential chemical elements such as potassium, calcium, magnesium, sodium and the phosphorus in natural proportions, water- and fat-soluble vitamins from milk such as A, B_1, B_6, C, E, and nitrogenous substances like whey proteins and free amino acids.

All that allow considering whey as a valuable nutrient media for propionic bacteria. The highest vitamin B_{12} yield for *Propionibacterium freudenreichii* cultivated on milk whey was 15 μg mL^{-1} (Berry & Bullerman, 1966) and that shows high application potential for enriching fermented products with natural vitamins.

Profitable production of various functional products with the use of milk whey enriched with B_{12} vitamin based on propionic fermentation can be arranged economically on the dairy factories. This can partially solve the problem of profitable utilization of milk whey which still remains one of the most serious problems of the modern world dairy industry.

Traditional ways of vitamin B_{12} microbiological production demand long-time multistage process of synthesis of the precursor and the vitamin itself, high-temperature processing for vitamin releasing from the cells, and also using of dangerous substances, as cyanides and the phenol for stabilization and extraction of vitamin, application of which at the food factories is inadmissible due to their high toxicity. Economically effective alternative to the common techniques of vitamin obtaining and stabilization can be ultrasonic treatment of the Propionibacterium cell culture developing directly in food product for the purpose of B_{12} synthesis intensification and soft destruction of bacteria cells without product overheating.

Low-frequency ultrasound was proved itself as a powerful and economic tool for intensification of biochemical reactions by means of increasing an exchange between cells and a nutrient medium (Christi, 2003), reducing duration of enzymatic hydrolysis and maintaining activity of some enzymes during cavitation (Rokhina et al., 2009).

For example ultrasound treatment with 20 kHz frequency which is widely used in food industry ultrasonic equipment increases growth rate and overall biomass of the cyanobacteriae cells (Francko et al., 1994). Treatment on this frequency is also used for

stimulation of anaerobic digestion processes in wastewater treatment plants (Dewil et al., 2006). Therefore low intensity ultrasound treatment of liquid medium which cause acceleration and initiation of chemical reactions in the sonicated medium can change the metabolic activity of bacteria cells (Akopyan & Ershov, 2005). By means of ultrasonic treatment it is possible to carry out the direct modulation of metabolic activity of bacteria for B_{12} vitamin yield increase without increasing in duration of production process using no special devices (Suchkova & Shershenkov, 2013).

MATERIALS AND METHODS

For the definition of ultrasonic processing influence on industrial strain of *Propionibacterium freudenreichii ssp. shermanii* metabolism bacteria cells were cultivated during 96 h in the nutrient media based on milk whey with concentration of lactose of 5% and adding of 0.002% $CoCl_2 \cdot 6H_2O$ and 0.001% $MgSO_4$ according to Zalashko (1990). The strain used for vitamin B12 production was industrial strain *Propionibacterium freudenreichii I-63,* which is a gram-positive, non-spore forming, rod-shape, and aerotolerant anaerobic bacterium producing vitamin B_{12} intracellularly.

The major problem in vitamin B_{12} production using *Propionibacterium* is the growth inhibition of the cell due to the accumulation of inhibitory metabolites such as propionic acid and acetic acid. Control of pH at approximately 7.0 is critical for reaching high vitamin B_{12} yields (Ye et al., 1996). Therefore pH was kept at level about 6.9 by means of periodic neutralization of the nutrient media during the cultivation by calcium carbonate.

Cell culture was treated by low intensity ultrasound at operating frequency of typical ultrasonic homogenizer 20 kHz within 10 and 20 min each 24 h.

First several cultivation cycles were performed in anaerobic conditions during all 96 h. Since oxygen is required for the biosynthesis of the lower ligand of active form of vitamin B_{12}, 5, 6-dimethylbenzimidazole, main product of this cultivation was vitamin analogs called corrinoids, which lack of nucleotide.

Next cultivation cycles were performed with continuous aeration of fermented media by means of sparging by sterile air during the second stage of cultivation after 48 h of anaerobic cultivation.

Measurement of titratable acidity of milk whey by 0.1N NaOH solution for definition of amount of organic acids synthesized by bacteria as primary metabolites was performed for research of metabolic activity of bacteria during the cultivation. Results are presented and compared in degrees of Turner (Table 1). Comparative photometric definition of corrinoid compounds concentration in the milk whey after cultivation was also made.

RESULTS AND DISCUSSION

It was found out that increase in duration of low intensity ultrasound treatment cause about 1–4% increase of total titratable acidity in all 20 minute treated samples and it depends upon duration of ultrasound treatment.

Table 1. Average total titratable acidity of fermented milk whey probes in various treatment conditions

Cultivation time, hours	Duration of treatment, min					
	0		10		20	
	ºT	%	ºT	%	ºT	%
0	3.1	100.0	3.1	100.0	3.1	100.0
24	40.6	100.0	41.6	102.5	39.5	97.3
48	74.9	100.0	74.9	100.0	74.9	100.0
72	108.9	100.0	111.0	101.9	113.0	103.8
96	140.7	100.0	140.4	99.8	146.4	104.0

That points on acceleration of fermentation processes and bacteria activity increase. Also changes in morphology of the bacteria cells subjected to ultrasound treatment and reduction of their sizes in comparison with untreated culture were observed. Average linear size of cells without treatment was 1.0–1.5 μm; linear size of treated cells in both experiments was 0.5–0.8 μm (Fig. 1).

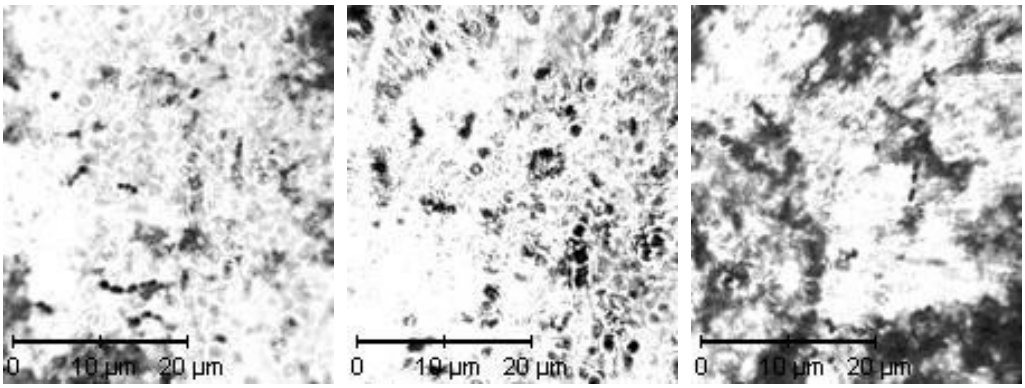

Figure 1. Microscope slides of fermented whey samples after anaerobic cultivation (untreated culture; 10 min treatment; 20 min treatment respectively).

Photometric analysis of fermented media was made after completion of fermentation process, thermal destruction of cells and filtration of the media. Received results are the same both for anaerobic cultivation and mixed anaerobic-aerobic cultivation cycles (Figs 2, 3).

During cultivation propionic bacteria use Co (II) from solution and turn it into corrin ring of cobalamines. Formed corrinoids have light absorption maximum which varies from 351 to 375 nm for various forms (Dolgov et al., 2004). Due to this fact it is possible to estimate their concentration in the fermented media and reveal the tendency of ultrasound impact on bacteria metabolic activity.

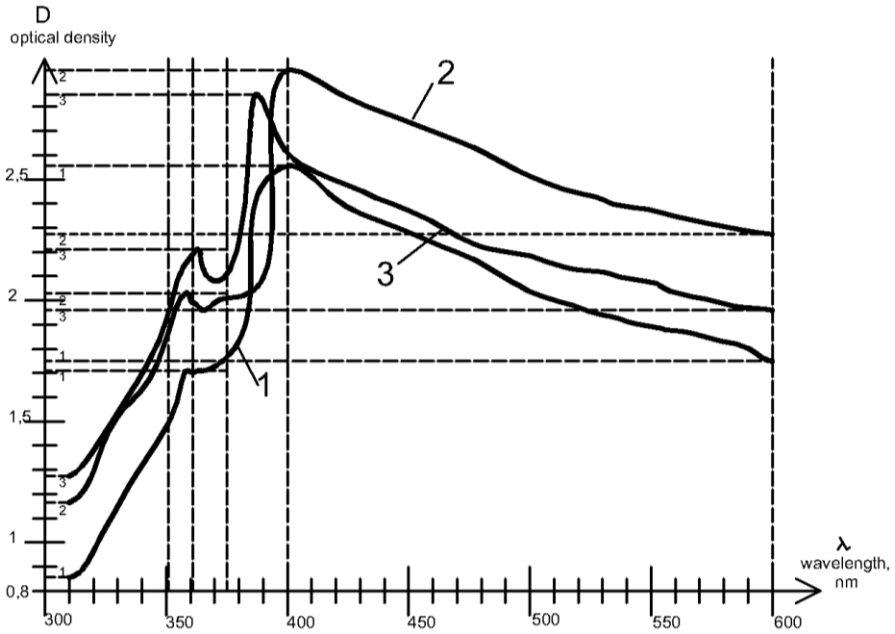

Figure 2. Absorption spectrum of fermented whey samples received after 96 h of anaerobic cultivation (1 is for untreated culture; 2 is for 10 min treatment; 3 is for 20 min treatment).

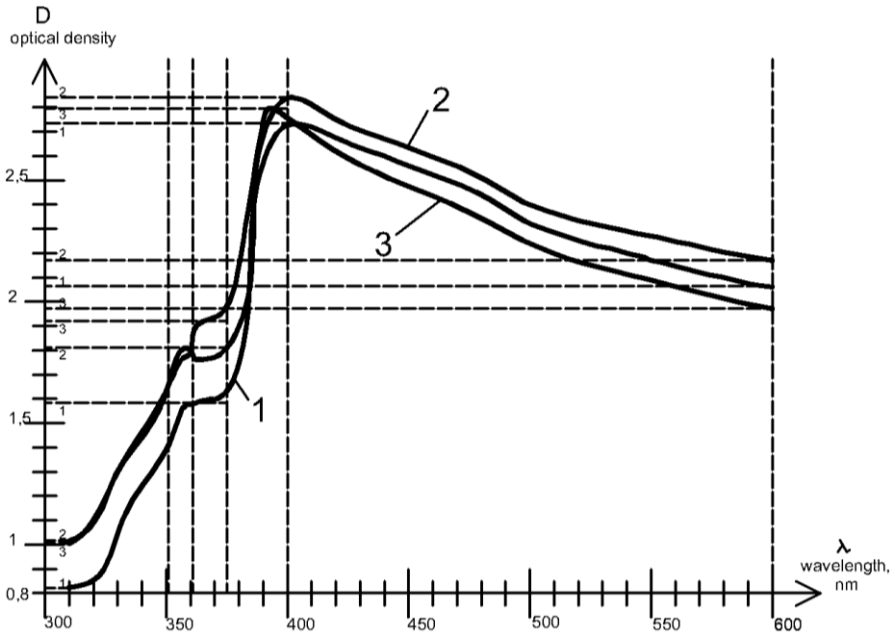

Figure 3. Absorption spectrum of fermented whey samples after 48 h of anaerobic and 48 h of aerobic cultivation (1 is for untreated culture; 2 is for 10 min treatment; 3 is for 20 min treatment).

For comprehensive analysis of corrinoid concentration graphical method based on additivity principle was used. For that purpose was measured difference between levels of 351–375 nm optical density peaks of averaged absorption spectra, received by comparing spectra of multiple probes, and overall optical density of probe averaged spectra without peak (Fig. 4). Results are presented and compared in relative photometrical units (Table 2).

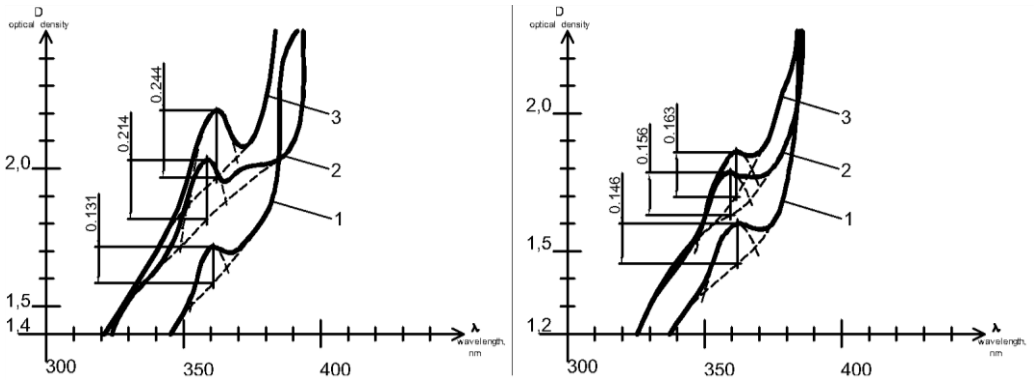

Figure 4. Graphical method for comparation of corrinoid concentration on fragments of absorption spectra of fermented whey samples.

Table 2. Comprehensive analysis of corrinoid concentration in fermented milk whey probes in various treatment conditions

Treatment duration, min	With aeration			Without aeration		
	0	10	20	0	10	20
Absorption maximum, nm	361	359	362	362	360	361
Overall optical density	1.717	2.032	2.211	1.601	1.786	1.860
Optical density without peak	1.585	1.817	1.967	1.455	1.630	1.697
Optical density difference	0.132	0.215	0.244	0.146	0.156	0.163
Content of corrinoids, photometrical units (D·100)	13.2	21.5	24.4	14.6	15.6	16.3
Relative content of corrinoids, %	100.0	162.9	184.8	100.0	106.8	111.6

Results show that formation of corrinoids is minimal without treatment and increase with increasing duration of ultrasound treatment from 10 to 20 min respectively.

Overall affect of the low-intensity ultrasound on metabolism of *Propionibacterium* can be noticed by comparing relations of left and right sides of different spectra and needs further investigation.

CONCLUSION

Received results show that ultrasonic modulation of a metabolism of propionic bacteria is the perspective direction for an intensification of B_{12} vitamin and its analogs obtaining and it can be also used in production of the various fermented food products enriched with B_{12} vitamin.

Received enriched milk whey can be used as an additive for production of the various dairy products enriched with the vitamin. Also there is an interest in the probiotic activity of *Propionibacterium freudenreichii* which can produce bifidogenic compound 1,4-dihydroxy-2-naphthoic acid and stimulates growth of bifidobacteria (Hugenshmidt et al., 2010). This effect can be also used in various fermented dairy products.

Enriched whey can be used for production of other enriched non-dairy like bakery products and confectionery for the purpose of ensuring the intake of daily requirement of B_{12} vitamin.

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REFERENCES

Akopyan, B.V. & Ershov, Yu. A. 2005. *Basics of ultrasound interaction with biological objects.* MGTU, Moscow, 224 pp. (in Russian).

Berry, E.C. & Bullerman, L.B. 1966. Use of cheese whey for vitamin B12 production. II. Cobalt precursor and aeration levels. *Appl. Microbiol.* **3**, 356–357.

Dewil, R., Baeyens, J. & Goutvrind R. 2006. Ultrasonic treatment of waste activated sludge. *Environ. Prog.* **25**, 121–128.

Dolgov, V.V., Ovanesov, E.N. & Schetnikovich, K.A. 2004. *Photometric analysis in laboratory practice.* Russian medical academy of post-graduate education, Moscow, 142 pp.
(in Russian).

Falentin, H., Deutsch, S.-M., Jan, G., Loux, V., Thierry, A., Parayre, S., Millard, M.-B., Dherbecourt, J., Cousin, F., Jardin, J., Siguier, P., Couloux, A., Barbe, V., Vacherie, B., Winker, P., Gibrat, J.-F., Gaillardin, C. & Lortal, S. 2010. The Complete Genome of Propionibacterium freudenreichii CIRM-BIA1^T, a Hardy Actinobacterium with Food and Probiotic Applications. *PLoS ONE* **5**, 1–12.

Francko, D.A., Al-Hamdani, S. & Joo, G.-J. 1994. Enhancement of nitrogen fixation in Anabaena flos-aquae (Cyanobacteria) via low-dose ultrasonic treatment. *J. Appl. Phycol.* **6**, 455–458.

Green, R. & Miller, J. 2010. *Handbook of Vitamins, 4th edition. 13. Vitamin B12*. CRC Press, 413–458, 608 pp.

Hugenschmidt, S., Miescher-Schwenninger, S., Gnehm, N. & Lacroix, C. 2010. Screening of a natural biodiversity of lactic and propionic acid bacteria for folate and vitamin B12 production in supplemented whey permeate. *Int. Dairy J.* **20**, 852–857.

Rokhina, E.V., Lens, P. & Virkutyte, J. 2009. Low-frequency ultrasound in biotechnology: state of the art. *Trend Biotech.***27**, 298–306.

Suchkova, E.P. & Shershenkov, B.S. 2013. Ultrasound modulation of Propionibacterium freudenreichii subsp. shermanii metabolic activity for production enriched by B12 vitamin

food products. *ESJ 'The processes and devices of food industry'*, **2**. (in Russian, English abstr.) http://processes.open-mechanics.com/articles/844.pdf

Ye, K., Shijo, M., Jin, S. & Shimizu, K. 1996. Efficient production of vitamin B12 from propionic acid bacteria under periodic variation of dissolved oxygen concentration. *J. Ferment. Bioeng.* **82**, 484–491.

Zalashko, M.V. 1990. *Biotechnology of milk whey utilisation.* Agropromizdat, Moscow, 192 pp. (in Russian).

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Comparative study on anti-oxidant activity of garlic grown in different regions

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Abstract. Currently reliance on natural products such as garlic and other vegetables is gaining popularity to combat various physiological threats including oxidative stress, cardiovascular complexities, cancer insurgence, and immune dysfunction. Garlic (*Allium sativum* L.) holds a unique position in history, belongs as a natural ingredient to different cuisines and was recognized for its therapeutic potential. Extensive research work has been carried out on the health promoting properties of garlic, often referred to its sulfur containing metabolites i.e. allicin and its derivatives. The aim of the present study was to compare garlic originated from different parts of the world (Chinese, Spanish, Lithuanian etc) on the basis of their anti-oxidant activity to evaluate their potential for different applications. As a result distinctive differences have been found in anti-oxidant activity of different garlic varieties. Garlic grown in Estonia possessed the highest antioxidant activity among the raw varieties. Black garlic has remarkable higher anti-oxidant content in comparison with other garlic samples.

Key words: anti-oxidant content, garlic, black garlic.

INTRODUCTION

Garlic (*Allium sativum* L.) is one of the bulbous rooty vegetables, which has been used for many centuries both as a flavouring agent and as a home remedy for the treatment of different health disorders. This vegetable is still mostly valued in different parts of the world due to its content of various flavouring components mostly presented by sulfur-containing chemical compounds. It has been shown that garlic aroma character is determined by the presence of di-2-propenyl disulfide (Gorinstein et al., 2005). Fortunately, garlic is also amongst a number of herbs having strong anti-microbial properties (Vokk, 1996). In recent decades application of garlic in phytotherapy has been widely investigated (Omar et al., 2007; Gorinstein et al., 2010).

It is well known that aging garlic at a constant temperature and humidity without adding any additives to a raw material, increases polyphenol compound presence. Thus, aged black garlic should exert stronger anti-oxidant effect compared to raw garlic, without decreasing the original effectiveness of the garlic. Gorinstein et al (2005) have shown that the bioactive compounds, electrophoretic patterns, and anti-oxidant potential of fresh Polish, Ukrainian and Israeli garlic samples are comparable but garlic samples subjected to heating at 100°C during 20 minutes preserve their bioactive compounds and anti-oxidant potential.

Even for the recommendations given by the physicians who wish to offer a patient herbal option for anti-microbial and/or anti-oxidative agents having several options to choose from.

MATERIALS AND METHODS

Physico-chemical and anti-oxidative measurements were performed to study garlic varieties grown in different climate conditions to compare their anti-oxidant activity.

The garlic samples were harvested in 2012–2013, the garlic bulbs were clean, white or greyish and well cured, the cloves were firm to the touch. The Estonian garlic was purchased from the producer Kadastiku in South Estonia. Other samples were purchased on the local market.

Black garlic (in the present work was used processed Spain garlic harvested in 2012) was also included into the study and the material was produced in 2013 by FS Baltic. Black garlic was produced by method of aging garlic, which in an embodiment involved the following procedures: classifying the garlic according to its condition and pre-treating it to achieve clean appearance; sealing the garlic in a vinyl pack by 10 kg and storing it in a tray; putting said tray in an aging device for a black garlic, applying steam and heat for 1–3 h while maintaining a temperature of 80–100°C inside the aging device, and treating by steam under a high temperature and high humidity condition; main aging by applying steam and heat to the treated garlic for 198 h while maintaining a temperature of 72–78°C inside the aging device; after-aging by applying steam and heat to the garlic, which had undergone main aging process, for 35 h while maintaining 60–69°C temperature inside the aging device; drying the after-aged garlic for 51 h while maintaining a temperature of 50–58°C inside the aging device; and low temperature after-aging by cooling obtained dried garlic at low temperature for 168 h while maintaining 0–5°C temperature inside the aging device. Provided here the method of aging black garlic characterized by drawing optimum time and condition without taking the storage condition, size, etc of the garlic (US Patent No US20110129580).

Sample preparation was performed as follows: 20 g of garlic was pounded with a pestle in a mortar until no garlic structure was recognizable any more (during a minute). To 0.5 g of disrupted material 20 ml of water (for the water soluble anti-oxidant measurements or 20 ml of methanol for lipid soluble anti-oxidant measurements) was added, shaked vigorously on Fortex and centrifuged 2 minutes at 13,200 rpm. 1 ml of supernatant was taken from both samples to perform anti-oxidant measurements. All the measurements of anti-oxidant activities were perfomed with the aim on Photochem, Analytik Jena. Preparation of the calibration curve was performed as described in the standard kit protocol ACL and ACW using volumes as displayed in the schemes in Manual (Photochem, Manual).

According to the principle of the ACW and ACL measurement method the free radicals were produced by optical excitation of a photosensitizer substance. Those radicals were partially eliminated from the sample by reaction with the anti-oxidants presented in the sample. In the measuring cell the remaining radicals caused the detector substance to luminescence and thereby the anti-oxidant capacity of the sample is determined. The anti-oxidative capacity of the sample was quantified by comparison

with the standard (constructed calibration curve with ascorbic acid or Trolox).

At least four parallels were performed. All samples needed dilution and the dilution rate was taken into account for final calculations. The results were given as ascorbic acid equivalent for water soluble anti-oxidants and Trolox equivalent for lipid soluble anti-oxidants in all investigated samples.

RESULTS AND DISCUSSION

In all garlic samples the moisture content has been determined by Halogen Moisture Analyzer and the results are given in Tabel 1.

Tabel 1. Moisture content of different garlic varieteis

Origin and year of harvesting	Moisture content, %
Estonian 2012	57.8
Chinese 2012	67.61
Spanish2012	37.66
Lithuanian 2013	63.17
Polish 2013	59.48
Chinese 2012 in a set	54.35
Black garlic 2013	45.37

Moisture content of different garlic samples varies significantly in the range from 37.66 to 67.61%. Moisture content was taken into account for further calculations of anti-oxidant acitivity.

In Tabel 2 all the calculations concerning the water soluble anti-oxidants and lipid soluble anti-oxidants are given per 1 g of dry weight of the product and also per 1 g of the product itself.

Tabel 2. Anti-oxidant activity of garlic samples (ACW – water soluble anti-oxidants and ACL – lipid soluble anti-oxidants)

Sample origin	ACW expressed as ascorbic acid		ACL expressed as Trolox equivalent	
	μmol $1g^{-1}$ dry weight	μmol $1g^{-1}$ product	μmol $1g^{-1}$ dry weight	μmol $1g^{-1}$ product
Estonian 2012	524.07	221.16	3.35	1.41
Chinese 2012	85.02	27.54	4.32	1.40
Spanish2012	14.05	8.76	2.40	1.50
Lithuanian2013	30.46	11.22	8.34	3.07
Polish 2013	16.94	6.86	11.32	4.59
Chinese 2012, in a set	84.25	38.46	6.51	2.97
Black garlic 2013	1,082.92	591.60	7.96	4.35

As a result distinctive differences have been found in anti-oxidant activity of different garlic varieties. Over 90 times higher anti-oxidant content in Spain garlic has been observed after aging process. Lipid soluble anti-oxidant content had less

remarkable differences. Would be interesting to produce black garlic from Estonian garlic expressing very high anti-oxidant content in a raw material. Over the past decades the application of herbs and other natural products has gained popularity, mint, garlic, pumpkin and beetroot amongst them. Garlic is known as a potent cardiovascular agent (Capasso, 2013). Therefore, garlic is a proper supplement in anti-atherosclerotic diets (Gorinstein et al., 2005). Latest investigations have given information about a possible role of black garlic in treatment of type 2 diabetes mellitus (Lee, 2009).

CONCLUSIONS

Garlic has been studied as one of the vegetables possessing comparatively high anti-oxidative activity. However the garlic bioactive value differs depending on the region, as it has been shown in the present investigation. It should be interesting to estimate different cultivars grown in the same region under the similar climatic conditions. Black garlic has very high potential as the source of anti-oxidants and it could be explained by changes in the chemical composition during the aging process.

REFERENCES

Capasso, A. 2013. Anti-oxidant action and therapeutic efficacy of *Allium sativum* L. *Molecules*, **18**(1), 690–700.

Gorinstein, S., Drziewiecki, J., Leontowicz, H., Leontowitcz, M., Naiman, K., Jastrzebski, Z., Zachwieja, Z., Barton, H., Shtabsky, B., Katrich, E. & Trakhtenberg, S. 2005. Comparison of the bioactive compounds and anti-oxidants potentials of fresh and cooked Polish, Ukrainian, and Israeli garlic. *J. Agric. Food. Chem.,* **53**, 2726–2732.

Gorinstein, S., Leontowicz, H., Leontowicz, M., Jastrzebski, Z., Najman, K., Tashma, Z., Katrich, E., Heo, B., Cho, J., Park, Y. & Trakhtenberg, S. 2010. The influence of raw and processed garlic and onions on plasma classical and non-classical atherosclerosis indices: investigations *in vitro* and *in vivo*. *Phytother Res*, **24**, 706–714.

Lee, Y., Gweon, O., Seo, Y., Im, J., Kang, M., Kim, M. & Kim, J. 2009. Anti-oxidant effect of garlic and aged black garlic in animal model of type 2 diabetes mellitus. *Nutr. Res. Pract.*, **3**(2), 156–161.

Omar, S.H., Abshar, U.H. & Nehal, M. 2007. Anti-carcinogenic and anti-tumorigenic effect of garlic and factors affecting its activity: a review. *Pharmacogn Rev.*,**1**, 215–221.

Photochem. Manual. Analytik Jena AG, 2003,2, 79

Vokk, R. & Loomägi, T. 1996. Anti-microbial properties of onion and garlic preparations. *Proc. Estonian Acad. Sci. Biol.*, **45**, ½: 68–72.

US Patent US201110129, 2009, published in 2011.

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Liposomal beta-carotene as a functional additive in dairy products

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Abstract. Encapsulation is of growing use in food technologies for various bioactive compounds. The study addresses the aspect of possible improved physiological activity that encapsulated form can enable. Liposomes obtained by dehydration/rehydration method were used as carriers of beta carotene. The liposomal additive was used for fortification of low-fat milk drink. Formation, structure and stability of the liposomes were controlled in the product matrix by transmission electron microscopy. Fraction-dispersed composition and aggregate stability of liposomal particles were investigated by means of dynamic light scattering. Liposomal beta-carotene was found to maintain stability during storage at 4 ± 2ºC within 15 days. Hexobarbital sleeping time test performed on mice fed with the product showed enhanced hepatostimulation effect achieved by the liposomal additive compared to free beta carotene. Liposomal beta-carotene is suggested to be used for development of functional health-promoting dairy products.

Key words: liposomes, beta-carotene, bioactive compounds, encapsulation, hepatostimulation activity.

INTRODUCTION

Dietary supplements are bioactive substances of natural origin for nutritional balance normalization and health promotion. Dietary supplements are the most efficient way of micronutrients deficiency elimination if their dosage corresponds to physiological requirements (Gichev & Gichev, 2009). Many dietary supplements contain substances of adaptogenic and restorative action, which stimulate body defense, enhance general resistance and vitality, physical and mental performance and reduce negative environmental effects and stress. Dietary supplements are the ingredients that provide health benefits beyond nutritional value, enhance physiological performance and prevent diseases (Paliyath et al., 2011).

Beta-carotene is a hydrocarbon with molecular formula $C_{40}H_{56}$ containing 11 conjugated double bonds. It is a biologically active substance playing important role in vital functions (pro-vitamin A). Beta-carotene is also an antioxidant involved in free radical defense. Fortification of food products is the most rational way of ensuring a

daily intake of β-carotene. However, beta-carotene is sensitive to environmental factors and rapidly loses activity being exposed to high temperature, light or oxygen (Krichkovskaya et al., 2001).

Combination of instability and hydrophobicity of crystal beta-carotene hampers its direct application in food processes. Development of acceptable β-carotene forms is required for introduction to food compositions. Encapsulation enables carotenoids stability optimization, dispersion in aqueous media and bioavailability improvement. The methods of microencapsulation of bioactives are used in medicamental compositions. Both natural substances (lipids, proteins, polysaccharides, etc.) and synthetic polymers (nylon, polyethylenimine, polylysine) are utilized as a wall material (Zuidam & Nedovic, 2010; Abbas, 2012).

Microencapsulation technology combines various physical and chemical methods including coacervation, precipitation, melt injection, fluidized bed coating, extrusion, vapor condensation, cross-linked polymers, polycondensation and polymerization. The method choice is mainly determined by properties of encapsulated ingredient (Sotnikov et al., 1999). For instance, the most complete encapsulation of flavorings can be achieved by spray drying. In this method encapsulation composition includes water-dispersed wall material, active ingredient, antioxidants and emulsifier (Merritt, 1981; Rich & Reineccius, 1988).

Preparation of water-dispersed forms of hydrophobic beta-carotene using microencapsulation includes several stages. Beta-carotene is dissolved in lipid fraction with emulsifiers and solvent at elevated temperature and pressure. Aqueous solutions of protectant colloid (gelatin or polymer) and softener (sugar) are prepared separately. The components are mixed and cooled down by pressure relieve. This results in formation of microparticles, namely, oil drops containing dissolved beta-carotene coated in protective shell. Fine powder is obtained after drying; it can be dispersed in water (Antoshkiw et al., 1982; Agapova et al., 1994).

Solubilization of beta-carotene can be achieved by using Tween 80 (polyoxyethylene sorbitan monooleate). Authors propose to use the solubilizate both for fatty and aqueous food systems (Sotnikov, 1995). Microemulsion 'Vetoron' produced in Russia includes alpha-tocopherol acetate, ascorbic acid and other bioactives in different proportions. There are successful results of using 'Vetoron' for medical applications (Sorokina & Lokshina, 2010). The possibility of using 'Vetoron' for fondant manufacture is currently investigated (Davidovich, 2009).

It should be noted that microencapsulates contain, as a rule, 1.5–10% of beta-carotene, up to 10% of stabilizer and antioxidants and 80–88.5% of non-ionogenic surfactants. In other words, they are mainly surfactants with beta-carotene addition and their combined biological effect requires special investigation (Shih, 2004). A way to obtain aqueous dispersion of beta-carotene at concentration of 80% requires application of extensive amounts of chlororganic solvents (dichlorethane, ethyl chloride, chlorophorm) or toluene (Bocharov, 2000). This makes application of this technique in food industry impossible (Zuidam & Nedovic, 2010).

Beta-carotene oil-in-water emulsions are prepared in few stages. At first stage β-carotene solution in non-polar solvent is added to vegetable oil at high temperature. This is followed by emulsification in aqueous phase containing stabilizer (Zuidam & Nedovic, 2010). Oil forms of beta-carotene increase its absorbtion *in vitro* and *in vivo*.

However, their use is limited and not applicable for products with reduced or zero fat (Berketova et al., 1995).

Liposomal encapsulation is a process that has been widely employed in medicine, pharmacology, cosmetology and genetic engineering (Lipowsky & Sackmann, 1995; Jesorka & Orwar, 2008; Mozafari et al., 2008). Liposomes are one of the most well studied supramolecular structures. There are researches on the diversity of lipid vesicles in terms of structure (size, number of layers), trapping capacity and localization in supramolecular structure (Gregoriadis, 2006). Liposomes can be objects of many applications because of independent biological activity of intact liposomes and enhanced efficacy of encapsulated ingredients (Bolshunova et al., 2010; Umnova, 2010). At present, liposomes are studied as an independent object in colloidal chemistry. They are considered as a target carrier of chemicals to cells, tissues and organs and as a model for biological membranes studies. However, their structure and properties still require investigation.

One of the promising liposomes applications is encapsulation of bioactive compounds and design of functional additives for food products. In the review by Gresler et al. (2008) liposomal technologies are considered as one of prospective lines in food industry. Benefits provided by embedding of active ingredients into liposomes include following: improved bioavailability of encapsulated ingredients; protection from environmental factors, e.g. oxygen, acids, processing temperature; ability to turn hydrophobic compounds into a water-dispersed form for better delivery; controlled release of an active ingredient (Mozafari et al., 2008).

The liposomes production processes known from literature could be adapted for the production of carotenoid-loaded liposomes as described by Ax (2003). In order to obtain the desired liposome size distribution, the dispersion is subjected to high-pressure homogenization, for example, by a microfluidizer.

The aim of this study was to show the possibility of designing a functional additive with beta-carotene in liposomal form and its usage for producing a dairy product. The additive should enhance biological activity and, therefore, improve health benefits of a product for daily consumption.

MATERIALS AND METHODS

Soya lecithin powder LeciPRO 90C (Orison Chemicals Ltd., China) was used as a liposomal wall material. Active ingredient for liposome encapsulation was crystalline beta-carotene (DSM Nutritional Products AG, Switzerland). Oil solution of α-tocopherol acetate 300 mg ml^{-1} (GalenoPharm, Russia) was used for antioxidant protection. Bioactive additive 'Vetoron' (Akvion, Russia) containing 20 mg ml^{-1} of beta-carotene and 8 mg ml^{-1} of vitamin E in water solution was used for preparation of non-liposomal carotene.

Carotene-loaded liposomes were prepared by dehydration/rehydration technique followed by mechanical homogenization (Weissig, 2010; Zabodalova et al., 2011). Soya lecithin was dissolved in approximately ten-fold n-hexane (Komponent-Reaktiv, Russia). Beta-carotene was dissolved in the minimal amount of chloroform (approximately 1 ml per 50 mg beta-carotene) and added to lipid solution to ensure the level of active ingredient being 0.5% (w/w) of lecithin. Antioxidant protection was achieved by addition of α-tocopherol in form of oil solution at the amount of 0.2%

(w/w) of lipid to the mixture. The blend was evaporated by the rotary evaporator EV311-V Plus (LabTech Inc., Italy) until lipid film was obtained at the flask bottom. Ethanol 50% solution was introduced to the dry film for better emulsification in the amount equal to soya lecithin weight, thoroughly stirred and the resulting mixture was left overnight. Next day the mixture was diluted with distilled water to ensure the lipid level of 10% (v/v). This mixture was an object of study as a water suspension of liposomes. The mixture was homogenized using mechanical mixer Biomix LE-402 (LaborMIM, Hungary) at the stirring rate of 15,000 rpm.

Empty liposomes were prepared in the same manner excluding the step of beta-carotene solution addition.

Reconstituted skim milk was used as a dairy product for fortification with liposomal beta-carotene. The skim milk powder contained 1% of fat (w/w), 36% of protein (w/w), 52% of lactose (w/w), 6% of ash (w/w) and 5% of water (w/w); it was obtained from the Bellakt Company (the Republic of Belarus). The skim milk powder was dissolved in distilled water (9 g skim milk powder per 100 ml distilled water) at 40–45°C. The mixture was left for 40 min for protein hydration and then pasteurized for 20−25 s at 76 ± 2°C. Liposomal additive was introduced into refrigerated milk with temperature of 4 ± 2°C in form of water suspension with 10% of lipid (v/v) and thoroughly stirred. Liposomal beta-carotene was added in the amount of 40% of daily adult requirement (5 mg) in beta-carotene established by the national health authorities (Rospotrebnadzor, 2009; Tutelyan, 2009) if consumed with 1 portion of the product (200 ml). Thus, the final levels of soya lecithin and beta-carotene in the product were 0.2 g and 1 mg per 100 ml of product, respectively.

Formation and internal structure of the liposomes were studied using electronic microscope JEM-100C (JEOL, USA). Two methods were used: negative contrast and ultra-thin slices technique. The first method was used as a preliminary to determine whether the desired structures were formed. Negatively stained samples were examined on support films with grids. The sample was mixed with a contrast solution. After 1 min of incubation, it was applied to the grid, the excess solution was drained, and the preparation was examined immediately (Lichtenberg et al., 1981; Podgorny & Ovchinnikov, 2002). Ultra-thin slices technique is more informative as it allows not only to determine the size, but also to obtain information about the internal structure. Ultra-thin slices were embedded in epoxy resin Araldite (Fluka, Switzerland) (Sora, 2007).

Fraction-dispersed composition and aggregate stability of liposomal particles were investigated by means of dynamic light scattering using spectrometer LKS-03 (Intoks, Russia). The method is based on scattered light intensity shift resulting from molecular movement in liquid. The technique provides information on particles size distribution. The percentage of diffused light corresponding to each fraction is related to surface area of diffusing particles which gives an indirect estimate of fraction share (Chernjavskij et al., 1985; Torchilin & Weissig, 2003). Since the dairy product matrix is too complex, fraction-dispersed composition of carotene-loaded liposomes was studied in the water suspension.

Titratable acidity and pH were controlled in the dairy product during storage to detect the effect of liposomal additive on milk stability. Titratable acidity was controlled according as follows: 10 ml of the product were mixed with 20 ml of distilled water and 3 drops of 1% phenolphthalein in a flask. The mixture was titrated

with 0.1 M NaOH until the defined pink color appeared. Titratable acidity was expressed as the volume of NaOH multiplied by ten (°T).

Beta-carotene concentrations in the dairy product and in the water suspension of liposomes during storage were determined using spectrophotometric method on UV-2,600 spectrophotometer (Shimadzu, Japan). The samples were diluted with distilled water to have 1% of fat (v/v). Beta-carotene was extracted from 1 ml aliquots of the dairy product or the water suspension using 2 ml of chloroform. Then 0.7 ml of the extract was collected and 3 ml of chloroform were added. The absorbance of carotenoid was measured in 1 cm cuvettes at a wavelength of 451 nm. Concentration was determined using standard curve (Dawson et al., 1989).

Biological activity of the dairy products was investigated using hexobarbital sleeping time test on white outbred mice (Dearing et al., 2006). Hexobarbital is a drug used for general anesthesia. It is degraded by microsomal enzymes in the liver that results in awakening. The test was performed on five groups with 10 animals in each (males, weight of 18–20 g). The dairy product samples were injected intragastrically in the dose of 0.2 ml per animal daily during 5 days. The last injection was made 30 min before hexobarbital injection. Hexobarbital was injected to all groups intraperitoneally in the dose of 60 mg kg^{-1}. The sleeping time was determined in seconds.

All measurements were performed at least in triplicate. Statistical analysis was performed using Microsoft Office Excel tools at theoretical frequency of 0.95.

RESULTS AND DISCUSSION

The liposomes characteristics

Fraction-dispersion composition and aggregative stability of liposomal dispersion in water and dairy product matrix were studied using transmission electron microscopy. Electron microscopy of the dairy product with carotene-loaded liposomes on the first day of storage proved the presence of continuous hollow spherical structures (Fig. 1). Unilamellar hollow vesicles and several multi-layered hollow structures can be distinguished clearly. The dark circles are probably slices of lipid drops with phospholipid and protein coatings (Gorbatova et al., 2011).

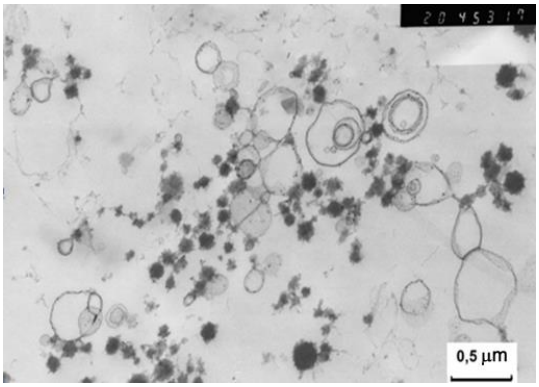

Figure 1. Electron microscopy image of ultra-thin slice of beta-carotene loaded liposomes in the dairy product matrix on the first day of storage.

The stability of lammelar structures of liposomes was studied in the dairy product during 10 days of refrigerated storage at 4 ± 2°C (Fig. 2). No structure disruption or aggregation of lipid vesicles were observed, however monolamellar liposomes dominate in the field of vision. Lipid drops tend to aggregate. This indicates the stability of liposomal additive in the dairy product during refrigerated storage for the time of observation.

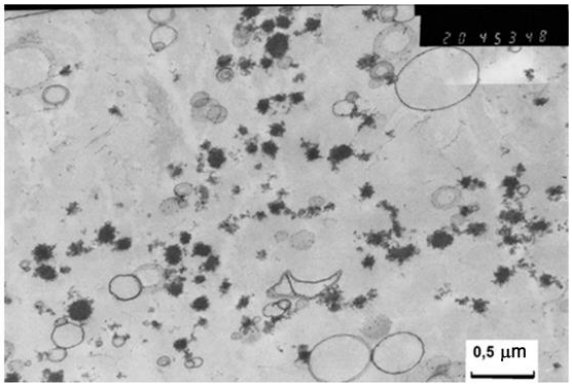

Figure 2. Electron microscopy image of ultra-thin slice of beta-carotene loaded liposomes in the dairy product matrix after 10 days of storage at 4 ± 2°C.

It should be noted that homogenization transfer of multi-layered liposomes to mono-layered ones and reduction of their mean diameter also occurs after mechanical stirring or sonication (Weissig, 2010).

Liposomes of different size and characteristics can be obtained depending on a preparation technique. The size is important in terms of liposomes application. Although the ultra-thin slices images can give a brief estimate of size distribution, it cannot be considered reliable as during sample preparation certain deformations of shape and structure occur.

More objective data on fraction-dispersed composition was obtained by means of dynamic light scattering (Table 1). It should be noted that the actual mean diameter is lower than the mean hydrodynamic diameter as measured by this method.

Table 1. Mean hydrodynamic diameter (d, nm) and percent of scattered light (%) of carotene-loaded liposomes in water suspension during storage at 4 ± 2ºC

Method	Storage duration, days					
	0		7		27	
	d, nm	%	d, nm	%	d, nm	%
without homogenization	1,100	12	–	–	–	–
	430	60	530	27	350	85
	126	28	220	63	115	11
with homogenization	17,500	30	21,000	40	22,000	12
	730	33	525	40	500	45
	260	33	170	19	190	42
	80	3	–	–	–	–

It is established that most of the particles in the solution have a size less than 1 μm. Different stereometry of lipids from the raw material may cause erratic distribution of formed lamellar particles size. Changes in particles size in the unhomogenized sample were not observed during the period of study (27 days at 4 ± 2ºC). It can be said that in this sample liposomes have stable sizes of about 0.5 μm or less. First fraction with the size of 1.1 μm could be formed by united particles which are subsequently separated.

The fraction with sizes of 17–22 μm was established in the water suspension liposomes samples subjected to the homogenization. Since there were no particles of this size in the original solution before homogenization, it can be assumed that this fraction is represented by a group of smaller particles joined together. This supposition is supported by this fraction part reduction from 30% to 12% in 27 days, which may be associated with the detachment of liposomes from aggregates formed during homogenization. Large fraction formation indicates the necessity of selecting another conditions or equipment for homogenization.

The products stability during storage

Stability of beta-carotene in liposome form during storage at 4 ± 2ºC was determined in two samples: the fortified dairy product and the water suspension of liposomes. Oxidized beta-carotene does not absorb at a wavelength of 451 nm. The oxidation rate of beta-carotene and the residual amount of non-oxidized beta-carotene in liposomes can be studied by the concentration in an extract from liposomes. The concentrations of beta-carotene in the chloroform extracts from the product and the suspension did not change during the observation period of 0–15 days and were 4.7 ± 0.5 μmol l^{-1} and 3.3 ± 0.7 μmol l^{-1}, respectively. This means that the liposomal beta-carotene is not exposed to oxidation.

The stability of the dairy product was evaluated by controlling titratable acidity and pH in reconstituted skim milk, in the product fortified with liposomal beta-carotene and in water suspension of liposomes. Both reconstituted skim milk with and without liposomal beta-carotene had pH of 6.75 ± 0.13 and titratable acidity of 18.9 ± 1.1°T for 0–13 days of storage at 4 ± 2ºC. Water suspension of liposomal additive had pH of 7.04 ± 0.09 for the same period and conditions of storage. Thus the liposomal additive did not affect pH and titratable acidity and did not lead to earlier spoilage of the product compared to the control sample.

Bioactivity of liposomal beta-carotene

Liposomal additive has a wide range of bioactivity due to its composition. The effect of the fortified dairy product consumption on the liver function of mice was chosen to study its bioactivity. The hexobarbital sleeping time test was performed using three samples: reconstituted skim milk, reconstituted skim milk fortified with liposomal beta-carotene and reconstituted skim milk fortified with water-soluble beta-carotene (‘Vetoron’). The two latter samples contained equal amounts of beta-carotene ensuring 40% of daily requirement of an adult – 1 mg%. The first control group received no treatment; the other control group received distilled water. The results are given in Table 2.

Table 2. The effect of the different products on hexobarbital sleeping time of mice

Group	Hexobarbital sleeping time	
	min	% of control
Control (intact animals)	40 ± 5	100
Distilled water	41 ± 4	100
Skim milk	39 ± 3	98
Skim milk + 'Vetoron'	30 ± 5	75
Skim milk + liposomal beta-carotene	24 ± 4	60

The data indicates decrease in hexobarbital sleeping time of animals treated with the samples fortified with beta-carotene. The sample with liposomal beta-carotene is more efficient than the one with water-soluble beta-carotene.

It should be noted that the lipids in liposomal wall bear not only structural function. Phospholipids also possess bioactivity and employed in the drugs stimulation of a liver function. An activation of liver detoxication function due to the functional additive intake was shown in the hexobarbital sleeping time test. This may indicate the ability of liposomes to penetrate hepatocytes and the effect of the functional additive on the liver detoxification rate (Shleikin et al., 2006). Further study is required to determine which components of the liposomal additive, jointly or separately, and in what form affect the liver function.

Liposomal beta-carotene has two active components: beta-carotene itself and lecithin as the wall material. Both contribute to hepatostimulation activity of the additive. However, it is not the only bioactive property. The additive can give a wide range of health benefits including anti-inflammatory, immunostimulatory activities, etc.

The results are in accordance with studies of other authors. Liposomal form doubles bactericidal and bacteriostatic activity of the phytochemical compositions as shown in the bioactivity comparison of their native and liposomal forms (Umnova, 2010). It was shown in the same research, that liposomal form of bioactives increases their membrane-stimulating properties by 40% and antioxidant activity by 25%. Liposomal form of *Bergenia crassifolia* leaves extract had a three times high immunomodulatory effect under cold stress as native extract (Bolshunova et al., 2010). Stimulating effect of the empty liposomes was also noted. Researches confirm liposomal form to be promising for phytocompositions and encapsulation of bioactives.

CONCLUSIONS

The novel functional additive for dairy products is proposed. The method used for liposome preparation is low-cost, simple and enables the production of unilamellar vesicles with the mean diameter below 1 μm. The stability of liposomes in dairy product matrix during storage is confirmed by transmission electron microscopy. The liposomal beta-carotene is not exposed to oxidation during storage for 15 days at 4 ± 2ºC.

The fortified low-fat milk drink was prepared with addition of liposomal beta-carotene to pasteurized reconstituted skim milk. The intake of 200 ml of the fortified product can supply 40% of daily requirement in beta-carotene. The liposomal additive

did not lead to earlier spoilage of the product as the product had pH of 6.75 ± 0.13 and titratable acidity of 18.9 ± 1.1°T for 0–13 days of storage at 4 ± 2ºC.

The activation of liver detoxication function due to the fortified dairy product intake was shown in the hexobarbital sleeping time test on mice. The contribution of each component of the liposomes in this bioactive property requires further study as well as other possible health benefits of the functional additive.

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REFERENCES

Abbas, S., Da Wei, C., Hayat, K. & Xiaoming, Z. 2012. Ascorbic Acid: Microencapsulation Techniques and Trends-A Review. *Food Rev.Int*. **28**(4), 343–374.

Agapova, E.V., Chichelnitsky, A.I., Mukhametshina, V.H., Spirichev, V.B. & Bukin Y.V. 1994. *Method of making dispersible in water β-carotene preparation.* RU Patent 2024505. (in Russian).

Antoshkiw, T., Cannalonga, M.A. & Guerin, F. 1982. *Stable carotenoid solutions.* US Patent 4,316,917.

Ax, K. 2003. *Emulsionen und Liposomen als Trägersysteme für Carotinoide*. [dissertation]. Univ. Karlsruhe, Karlsruhe, 192 pp.

Berketova, L.V., Semenova, I.A. & Agapova, E.V. 1995. Preservation of beta-carotene in beverage powders. *Voprosy pitaniia*, **1**, 12–14. (in Russian, English abstr.).

Bocharov, V.V. 2000. *A method for producing water-dispersable formulations of fat-soluble vitamins.* RU Patent 2159765. (in Russian).

Bolshunova, E.A., Lamazhapova, G.P. & Zhamsaranova, S.D. 2010. Research of liposomal form of *Bergenia crassifolia (L.) Fritsch* influence on formation of adaptation potencial of the body. *ESSUTM Bulletin*, **4**, 83–88. (in Russian, English abstr.).

Chernjavskij, V.A., Lebedev, A.D. et al. 1985. Study of factional disperse composition of vesicles from the total E. coli lipid at different incubation conditions by optical mixing spectroscopy. *Biologicheskie membrani*. **2**, 376–382. (in Russian).

Davidovich, E.A. 2009. *Study of beta-carotene preparation 'Verotron' influence on fondant sweets organoleptic characteristics.* Pishevaja i pererabativajushaja promishlennost, Moscow, 723 pp. (in Russian).

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. & Jones, K.M. 1989. *Data for Biochemical Research, 3rd Ed*.; Oxford University Press, Oxford, England, 580 pp.

Dearing, M.D., Skopec, M.M. & Bastiani, M.J. 2006. Detoxification rates of wild herbivorous woodrats (Neotoma). *Comp. Biochem. Physiol. A-Mol. Integr. Physiol*. **145**(4), 419–422.

Gregoriadis, G.E. 2006. *Liposome Technology, 3d ed. Vol. I. Liposome Preparation and Related Techniques.* Informa Healthcare, 346 pp.

Gichev, Y.Y. & Gichev, Y.P. 2009. *New guide on micronutrientology (biologically active additives to food and human health).* Triada-HB, Moscow, 304 pp. (in Russian).

Gorbatova, K.K., Gunkova, P.I., Belodedova, J.V. & Kolcova M.V. 2011. Fat globe membranes formation. *Molochnaja promishlennost*, **1**, 48–49. (in Russian, English abstr.).

Gresler, S., Gazso, A., Simko, M., Nentwich, M. & Fiderel, U. 2008. Nanopartikel und nanostrukturierte Materialien in der Lebensmittelindustrie. *Nano-trust Dossiers*, **004**, 1–5.

Jesorka, A. & Orwar, O. 2008. Liposomes: technologies and analytical applications. *Annu. Rev. Anal. Chem*., **1**, 801–832.

Krichkovskaya, L.V., Donchenko, G.V. & Chernenkaya, L.A. 2001. Biological activity of microbiological carotene after its stabilization by phenolic antioxidants. *Vestnik problem biologii I medicyni*, **1**, 109–112. (in Russian).

Lichtenberg, D., Freire, E., Schmidt, C.F., Barenholz, Y., Felgner, P.L. & Thompson, T.E. 1981. Effect of surface curvature on stability, thermodynamic behavior, and osmotic activity of dipalmitoylphosphatidylcholine single lamellar vesicles. *Biochemistry*, **20**(12), 3462–3467.

Lipowsky, R. & Sackmann, E. 1995. *Handbook of Biological Physics, Vol. 1: Structure and Dynamics of Membranes*. Elsevier, Amsterdam, 1020 pp.

Merritt, C.G. 1981. *Encapsulation of materials.* US Patent 4,276,312.

Mozafari, M.R., Johnson, C., Hatziantoniou, S. & Demetzos, C. 2008. Nanoliposomes and Their Applications in Food Nanotechnology. *J.Liposome Res*. **18**, 309–327.

Paliyath, G., Bakovic, M. & Shetty, F. 2011. *Functional Foods, Nutraceuticals and Degenerative Disease Prevention*. John Wiley & Sons, Inc., 403 pp.

Podgorny, G.N. & Ovchinnikov M.M. 2002. *Liposomes negative staining method for electron microscopic examination.* RU Patent 2195263. (in Russian).

Rich, S.J. & Reineccius, G.A. 1988. *Flavor Encapsulation*. American Chemical Society, Washington, 202 pp.

Rospotrebnadzor. 2009. *Norms of physiological requirements in energy and nutrients in various groups of population in Russian Federation. Methodological recommendations MR 2.3.1.2432-08.* Federal Center of Hygiene and Epidemiology, Moscow, 38 pp. (in Russian).

Shih, E. 2004. Effectiveness of vitamin-mineral complexes in terms of interaction of micronutrients. *Pharmaceutical Bulletin*, **358** (37), 1–6. (in Russian).

Shleikin, A.G., Golovkin, V.I. et al. 2006. Influence of olifen on the content of ethanol in blood. *Fundamentalnie issledovania*. **3**, 93–94 (in Russian).

Sora, M.C. 2007. Epoxy plastination of biological tissue: E12 ultra-thin technique. *J Int Soc Plastination*, **22**, 40–45.

Sorokina, N.V. & Lokshina, E.E. 2010. Use of beta-carotene for frequently ill children. *Voprosi prakticheskoi pediatrii*. **2**, 107–109. (in Russian).

Sotnikov, P.S. 1995. *Method of preparing a solution of beta-carotene and the composition based on beta-carotene for fortification and coloring foods.* RU Patent 2043339. (in Russian).

Sotnikov, P.S., Bykov, V.A. & Lazarev, M.I. 1999. *A method for producing a water-soluble vitamin preparation and a method for producing vitamin preparation.* RU Patent 2139935. (in Russian).

Torchilin, V.P. & Weissig, V. 2003. *Liposomes: A Practical Approach*. Oxford University Press, New York, 401 pp.

Tutelyan, V.A. 2009. Norms of physiological requirements in energy and nutrients in various groups of population in Russian Federation. *Voprosy pitaniia*, **78**(1), 4–16. (in Russian, English abstr.).

Umnova, O.A. 2010. *Comparison of the biological activity of phytochemical compositions in native and liposomal forms*. Moscow University Chemistry Bulletin, **65**(6), 397–402.

Weissig, V. 2010. *Liposomes: Methods and Protocols, Volume 1: Pharmaceutical Nanocarriers*. Humana Press, New York, 564 pp.

Zabodalova, L. A., Chernjavskij, V. F., Ishchenko, T.N. & Skvortcova, N.N. 2011. Production of liposomes from soybean lecithin. *Series Processes and Equipment for Food Production*, **2**. (in Russian, English abstr.). http://processes.ihbt.ifmo.ru/file/article/9246.pdf

Zuidam, N.J. & Nedovic, V.A. 2010. *Encapsulation Technologies for Active Food Ingredients and Food Processing*. Springer, New York, 400 pp.