Food Quality and Safety

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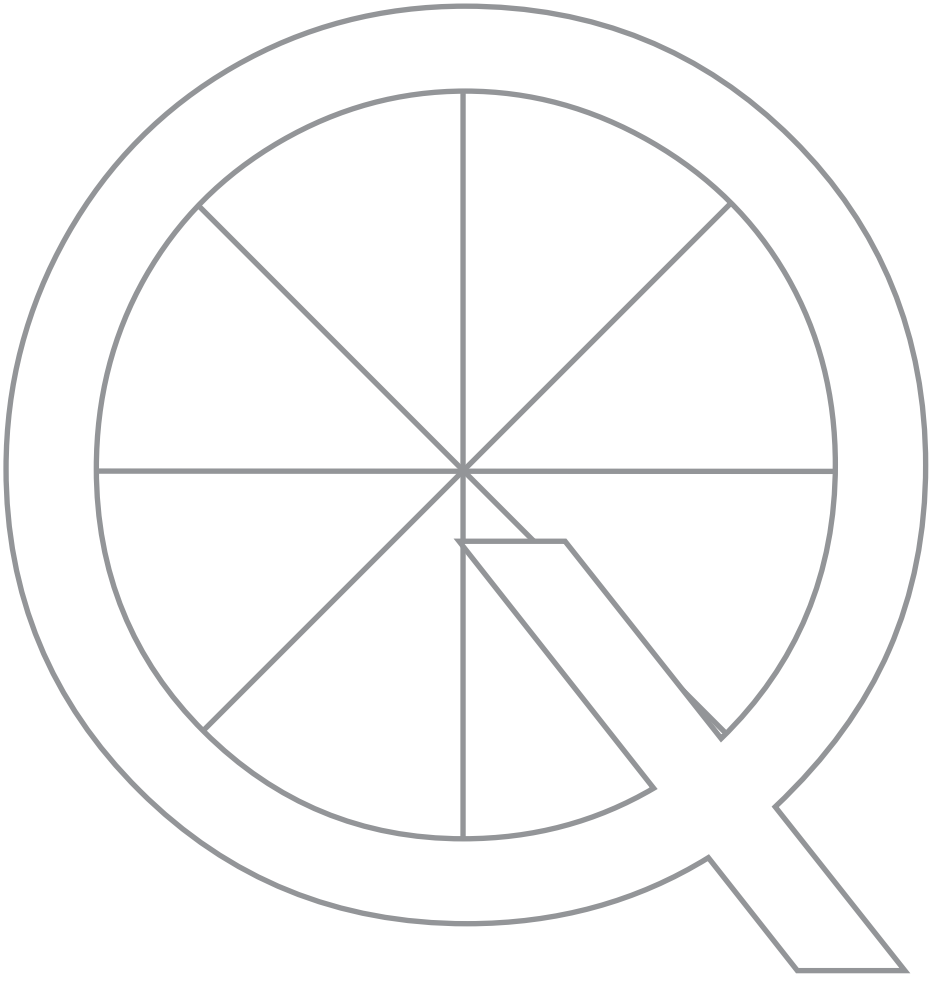

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PREFACE

The term "quality" is understood in many ways and depends on the type of product as well as on individual needs of the consumer. Generally, product quality are the quality characteristics that meet the expected needs of the consumers.

In case of food products, the widely accepted definition of quality includes such elements as pro-health qualities, sensory attractiveness and availability. The first of them concerns proper nutritional and caloric value and food safety. All required quality parameters of food products are created on the level of the primary production, i.e. plant production and animal breeding, proper handling of raw materials, processing and preservation technology, storing and applying good practices during distribution.

The quality of food products depends on all elements of environment in which production is conducted. Soil, water, air and feed contamination decide about the quality of plant and animal raw materials and may limit their use in food production. Food processing methods, food additives, preservation, packing and labelling technologies also decide about the health quality of food.

The producer is responsible for the quality and food safety. Legal regulations guarantee the safety of consumers. The Codex Alimentarius Commission, created by FAO/WHO, developed the Codex Alimentarius which defines the principles of the HACCP system as the basic tool guaranteeing safety in food production. The Codex Alimentarius contains guidelines for introducing legal regulations. The EU countries have adopted unified acts in the form of regulations, directives, decisions and recommendations which are introduced to national legal systems. The European Food Safety Authority, founded in January 2002, guarantees that the regulations and norms up to date and correspond to the scientific progress in research on food, its influence on human health as well as on food production methods.

The present study presents and summarises the most important results of research on food quality and factors determining the quality of food products including the following:

- obligatory and voluntary systems of food quality assurance,
- the quality of food material,
- research on the methods for analysing food quality,
- the possibilities of technological improvement of food quality,
- the quality of food available on the market.

The above research topics correspond to the demands of the whole food sector and to the expectations of food consumers.

Editors:
Grażyna Krasnowska
Anna Pęksa

Wrocław, 2009

CHAPTER 1

SYSTEMS GUARANTEEING SAFETY AND QUALITY IN FOOD PRODUCTION

1

FOOD QUALITY AND SAFETY: ROLE, DISSEMINATION AND ASSESSMENT OF CERTIFICATION SYSTEMS

Introduction

Food quality is a multi-faceted phenomenon including extrinsic – production system characteristics and environmental aspects – as well as intrinsic – product safety, health, sensory properties, shelf life, reliability and convenience – characteristics [Luning et al., 2002]. Markets for agricultural and food products are characterized by high information asymmetries since supply chain partners such as producers, processors and retailers are in most cases much better informed about the quality of their products than consumers [Henson & Traill, 1993]. Often consumers are not at all able to control important quality attributes such as food safety, nutritional value, organic production or region of origin. Such credence attributes can result in market failure due to a lack of credible information in the market [Akerlof, 1970].

Against this background, several attempts to reduce information asymmetries and to better inform consumers have been made. These attempts include regulatory approaches. Food laws can be traced back to ancient times. Since trade of agricultural and food products was one of their main income sources and crucial for the supply of the local population, antique as well as medieval towns laid down regulations on food quality, food inspections, and metrics and weights [Mettke, 1979]. In many countries the late 19th century marked a starting point for a much more systematic and comprehensive regulation of food quality based on more advanced natural science knowledge and improved analytical methods. More recent decades have been characterized by efforts to internationally harmonize food law [Kastner & Pawsey, 2002]. This also includes extensive legislation by the European Union (EU) in recent years. General Food Law Regulation (EC) 178/2002 has strongly contributed to the ongoing international harmonization trend, for instance by providing definitions of technical terms such as food, feed or placing on the market. Furthermore, the General Food Law Regulation has also introduced several new principles of food safety legislation such as the "from farm to fork" approach [Streinz, 2007].

Besides legislation, certification systems can be considered a second important approach to improved food safety and quality, reduced information asymmetries and better informed consumer choices. During the last decade a large number of very diverse certification standards have been established. In this paper we aim at systematically analyzing the role, dissemination and assessment of certification systems in food supply chains.

Certification Systems in Food Supply Chains: Roles and Dissemination

"Certification is the (voluntary) assessment and approval by an (accredited) party on an (accredited) standard" [Meuwissen et al., 2003]. A key feature of certification systems is that inspections are carried out by independent bodies beholden to standards laid down by external organizations [so-called third-party audits; Luning et al., 2002]. By means of regular control and – whenever necessary – additional unheralded checks, neutral inspection institutions, in many cases private auditing companies, monitor those parts of food supply chains that are captured by the respective certification system. After successfully passing the auditing procedure, farms and/or firms are awarded a certificate that can be used as a quality signal in market relationships. This has interesting implications for the organization of food supply chains. System participants, for instance certified farmers, do not enter contractual relationships with processors but remain independent and can sell their products on open markets [Theuvsen, 2009]. Due to the reduction of quality uncertainties by certification systems, spot market transactions are favoured over alternative ways of organizing food supply chains [Schramm & Spiller, 2003].

Certification schemes have become widely prevalent in agriculture and the food industry. In Germany alone about 40 different such schemes are used for certifying farms and firms. For the EU, the number of more than 380 certification schemes is sometimes cited [Wesseler, 2006). A closer look at the certification systems in place reveals a broad spectrum that can be organized along different dimensions [Theuvsen & Spiller, 2007; Theuvsen et al., 2007]:

- Target: Consumer-oriented schemes such as organic farming, fair trade and PDO, PGI and TSG schemes can be distinguished from business-to-business tools such as GlobalGAP, the International Food Standard, the BRC Global Standard or ISO 9001 and 22000.
- Focus: Many schemes focus on process characteristics with regard to basic production and documentation standards (e.g., IKB in the Netherlands or the British Assured Farm Standard), improved animal welfare standards (for instance Freedom Food in the United Kingdom) or more environment-friendly and sustainable production (organic farming schemes). Other schemes focus on product characteristics such as region-of-origin (PDOs and PGIs) or traditional character (for instance, the Dutch TSG Boerenkaas).
- Goal: Basic level schemes try to guarantee compliance with legal and other minimum requirements in mass markets (for instance, GlobalGAP, Certus in Belgium, IKB in the Netherlands and Qualität und Sicherheit in Germany) whereas marketing oriented approaches deal with niche markets and product differentiation (for instance, PDOs, PGIs, Freedom Food and organic farming schemes). The latter typically rely on labelling to inform consumers willing to pay for special process or product characteristics, whereas in the case of the mass marketing approach labels are a rare exception (see for example the German system 'Qualität und Sicherheit').
- Content: Quality assurance schemes can focus on safety (IKB, IFS etc.), quality (for instance PDO schemes), or production standards (e.g. organic farming standards).

- Number of stages of the food supply chain involved: Standards for one stage of the supply chain involve only direct suppliers and customers, for instance food manufacturers and retailers in the case of the International Food Standard or farmers and retailers in the case of the British Assured Farm Standard. On the other hand, chain-wide concepts include all stages of the food supply chain. The German Qualität und Sicherheit GmbH, for instance, was founded by associations representing all stages of the meat supply chain (mainly feed production, agriculture, slaughtering, processing and retailing).
- Standard owner: A wide range of schemes can be differentiated according to the respective standard owner. The spectrum embraces completely state-run systems (like organic farming in Denmark), governmental schemes with private inspections (e.g., organic farming schemes in most European countries), systems developed by international standardization organizations (e.g., ISO 9001 and 22000), stakeholder approaches (e.g., Word Wildlife Fund/WWF in the case of Marine Stewardship Council; Fairtrade), schemes founded by producer associations (e.g., farmers associations in the case of the British Assured Farm Standard), standards by private inspection bodies (e.g., Vitacert by the German Technical Monitoring Institution/TÜV) and retailer driven schemes (e.g., BRC Global Standard and International Food Standard).
- Degree of national adaptation: Original schemes (for instance, GlobalGAP) are sometimes used as blueprints for national adoptions of a broader approach (e.g. ChileGAP, KenyaGAP).
- Geographic focus: With regard to the geographic focus regional approaches (for instance quality assurance systems developed and run by the German federal states such as Geprüfte Qualität Bayern in Bavaria), national systems (e.g., Certus in Belgium and KLASA in the Czech Republic), international approaches (e. g., the French-German International Food Standard or the European regulation on organic farming) and global standards, e.g. GlobalGAP, can be distinguished.
- Number of participating firms: Small regional or highly differentiated schemes often have only several dozen or a few hundred participants. Medium-sized schemes have several hundred to several thousand members (for instance, about 3,200 farms and firms in the case of the Demeter organic farming scheme) whereas large standards often have ten thousands of members (e.g. more than 100,000 farms and firms in the case of the German Qualität und Sicherheit system).

The distinctions listed above show that certification systems have various roles in food supply chains. Some systems aim at informing consumers whereas others are used in order to reduce quality uncertainties in business-to-business relationships. Some systems have been implemented to advertise local (for instance, Bavarian) products, whereas others functions as door openers for farms and firms in developing and transitional economies willing to enter international food supply chains. Whereas minimum requirement standards mainly contribute to the reduction of food safety risks, differentiation schemes aim at triggering consumers' willingness to pay for higher product or process qualities. Recent developments such as labels indicating the sustainability standards of food products, food miles labels and carbon footprint labels show that increasingly more roles are attributed to food product labels. Nonetheless, their basic role is always the same: providing additional information on product or process characteristics to supply chain partners or consumers and, thereby, reducing information asymmetries. To what extent this goal is accomplished depends on the criteria the certification systems sets and its reliability and credibility. The latter very much

depend on the qualification and independence of auditors, the incentives provided to auditors and participants by the certification system, the intensity of controls and the control technology applied (for instance, database-supported checks of the reliability of external audits) [Jahn et al., 2005].

Certification systems have a long history in the Mediterranean countries in Europe. The Consorzio del Grana Tipico in Italy, for instance, which controls the Grana Padano PDO was founded in 1928 [www.granapadano.com]. Nowadays certification systems are also widely prevalent in Western and Northern Europe where – unlike in Southern Europe – minimum requirement standards such as IFS and IKB play a dominant role. In recent years a rapid internationalization of many certification systems has been going on [Gawron & Theuvsen, 2009b]. GlobalGAP, for instance, is currently active in more than 80 countries on every continent [www.globalgap.org). It can also be observed that certification systems once established in Western Europe are gaining more and more relevance in Central, Eastern and South-Eastern Europe due to the growing role these countries play in European food supply chains. Figure 1 shows the number of selected certificates conferred in Central, Eastern and South-Eastern Europe in 2008. Compared to Southern and Western Europe, these countries are still in a catch-up process with regard to certification systems.

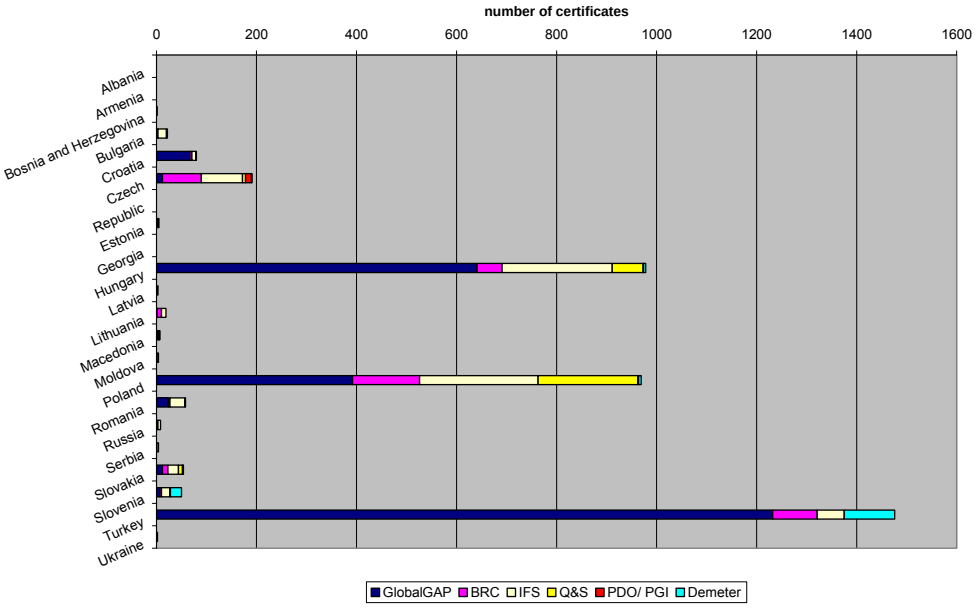

Fig. 1. Number of certificates conferred in Central and Eastern Europe [Gawron & Theuvsen, 2009b]

The international dissemination of certification scheme has gained much momentum. Nonetheless, it is still vividly discussed whether certification systems must be considered as potential barriers to or catalysts of the participation of transformational and developing economies in international trade with agricultural and food products [Anders & Caswell, 2009]. Regardless of this on-going discussion, remarkable differences between the Central, Eastern and South-Eastern European countries surveyed by Gawron and Theuvsen (2009b)

can be observed. A quick glance at export statistics indicates that high numbers of certificates coincide with high exports. Turkey, Poland, Hungary and the Czech Republic are good examples of the hypothesis that quality certificates have become a *conditio sine qua non* for successful exports of agricultural and food products. This observation parallels similar results from South America [Lazo et al., 2007]. Furthermore, with the exception of Turkey, admission to the EU seems to support the diffusion of quality assurance schemes. This also nourishes the hypothesis that certificates, at least in some cases, function as non-tariff trade barriers on international markets [Zheng & Jiang, 2002].

An interesting but so far hardly analyzed question is to what extent cultural differences between regions affect the international dissemination and implementation of certification systems. These systems in most cases represent Western bureaucratic management styles that are hardly adapted when being exported to culturally different regions in the world. It can be hypothesized that important cultural dimensions such as power distance, individualism/collectivism, masculinity/femininity, uncertainty avoidance and long- and short-term orientation [Hofstede & Hofstede, 2006] strongly influence the implementation of certification systems [Gawron & Theuvsen, 2009c].

Assessment of Certification Systems in Food Supply Chains

Due to various driving forces, major parts of the agri-food sector have been certified. Nonetheless, the implementation of certification systems has evoked controversial discussions. Whereas some authors consider the introduction of chain-wide certification systems an important contribution to implementing an efficient operational quality management system and gaining and sustaining competitive advantages, many farms and firms still feel incapacitated by the strict regulations imposed by these systems and consider them more or less a useless bureaucratic workload and ineffective "paper tigers" [Curkovic & Pagell, 1999; Theuvsen, 2005]. Some authors even regard the widespread implementation of certification systems as a management fashion and a reaction of agribusiness firms to institutionalized pressures in their environments [Beck & Walgenbach, 2002; Walgenbach, 2007].

Against this background, more and more in-depth analyses of the efficiency and effectiveness as well as of the proper design of certification systems in the agribusiness have been published [Canavari & Spadoni, 2004; Theuvsen & Peupert, 2004; Jahn et al., 2005; Lazo et al., 2007; Enneking et al., 2007; Schulze et al., 2008]. More recent studies have also investigated the assessment of certification schemes by supply chain partners. Since the assessment of certification systems is decisive not only for farms' and firms' willingness to voluntarily participate in those systems but also for their motivation to comply with the standards laid down, it seems worthwhile to take a closer look at the evaluation of certification systems by agribusiness firms.

In 2005 Gawron & Theuvsen (2009a) surveyed 65 IFS-certified German food manufacturers. The International Food Standard (IFS) has gained much relevance in the European food industry since all major German and French retailers have subscribed to the system and usually do not accept suppliers that do not have IFS certificates. Therefore, the IFS has evolved as a widely accepted certification standard not only for private labels but also for manufacturer brands. It has largely replaced the ISO standard in the German and other countries' food industry. The IFS is divided into four parts: the IFS Protocol, the Catalogue

of Requirements, the Requirements for Certification Bodies and Auditors and the IFS Report [Buhlmann et al., 2004]. It widely parallels earlier standards such as the ISO 9001 standard and the BRC Global Standard. Its main technical chapters are management of the quality system, management responsibility, resource management, product realization, and measurements, analysis and improvement (Figure 2).

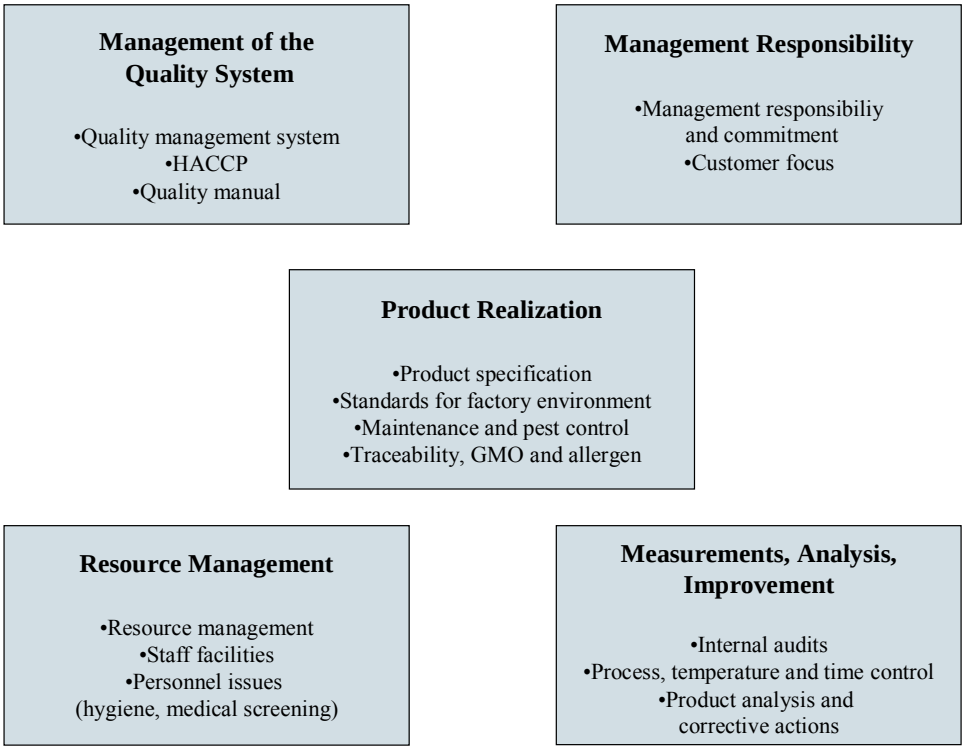

Fig. 2. Technical chapters of the IFS Standard [Gawron & Theuvsen, 2009a]

In April and May of 2005, 65 IFS-certified food suppliers were interviewed by telephone using a standardized questionnaire with sixteen questions. The persons interviewed could express their attitudes by evaluating statements on five point Likert scales from -2 ("I do not agree at all.") to +2 ("I fully agree."). The interviews then mainly focused on the perceived advantages and disadvantages of the IFS. A pre-test showed that the questionnaire was exhaustive and that the target group did not experience any difficulties with the questionnaire.

The companies interviewed represent eleven different agribusiness subsectors including confectionery and snacks (12 companies), meat processing (11), milk processing (10), and bakery products (9). Of the respondents 45.6 percent employ one to five hundred employees; however, there are also companies with more than three thousand employees in the sample. All the companies interviewed are private label manufacturers. The percentage of private label production is below 25% in 24.5 percent of the responding companies,

between 25 and 50% (34% of respondents), between 51 and 75% (24.5%), or above 75% (17%). 17% of the companies surveyed have foreign subsidiaries; the other respondents have only domestic production.

Figure 3 shows that the respondents perceived several advantages of the IFS. The statements that the IFS requirements are well structured ($\mu = 0.74$; $\sigma = 1.136$) and that other standards can be accomplished at the same time ($\mu = 0.70$; $\sigma = 1.136$) received the strongest support; 72.3% and 68.7%, respectively, of the respondents agree or fully agree with these statements. Furthermore, the contribution of the IFS to a continuous improvement process is generally appreciated; 68.8% of the respondents agree or fully agree ($\mu = 0.80$; $\sigma = 1.237$). In contrast to this, positive effects on internal business processes ($\mu = 0.50$; $\sigma = 1.113$), quality motivation ($\mu = 0.46$; $\sigma = 1.300$) and external logistics ($\mu = 0.16$; $\sigma = 1.280$) are only somewhat rarely reported. Nonetheless, high standard deviations indicate a broad spectrum of diverse attitudes in the sample. Whether or not the implementation of the IFS decreases the number of external audits is discussed controversially by the respondents. While 43.1% of the respondents agree or fully agree that the IFS is able to reduce the number of audits, 44.8% do not think this aim can be achieved. A slightly negative mean value ($\mu = -0.12$) and a high standard deviation ($\sigma = 1.568$) clearly show the very diverse opinions in the food processing industry concerning the effect on the number of audits. Furthermore, a reduction of the certification costs is not expected by a large majority ($\mu = -0.85$; $\sigma = 1.311$).

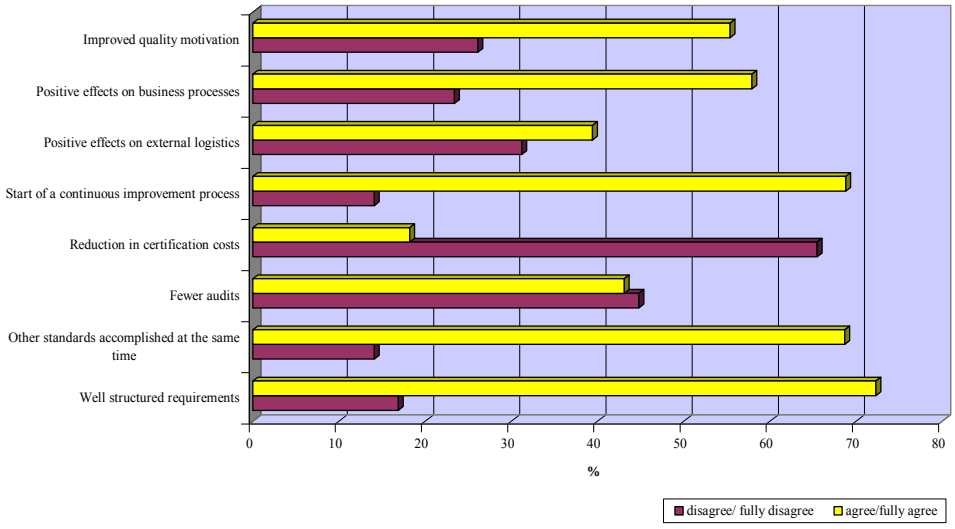

Fig. 3. Perceived advantages of the IFS [Gawron & Theuvsen, 2009a]

As one can see from Figure 4, the strict requirements of the IFS are perceived as the main disadvantage; 69.3% of the respondents agree or fully agree ($\mu = 0.77$; $\sigma = 1.196$). In contrast to this, the statement that the IFS will reduce certification costs finds only mixed support. One group of respondents agree with the statement, whereas another group of nearly the same size disagree or even strongly disagree ($\mu = 0.13$; $\sigma = 1.409$). Other disadvantages, such as a possible lack of reasonability ($\mu = -0.69$; $\sigma = 1.030$) and comprehensi-

bility of requirements (μ = -0.72; σ = 1.111), low action orientation (μ = -0.42; σ = 1.130) and low managerial practicability (μ = -0.58; σ = 1.029) were rejected by the majority of the respondents. Nonetheless, high standard deviations indicate contradictory opinions.

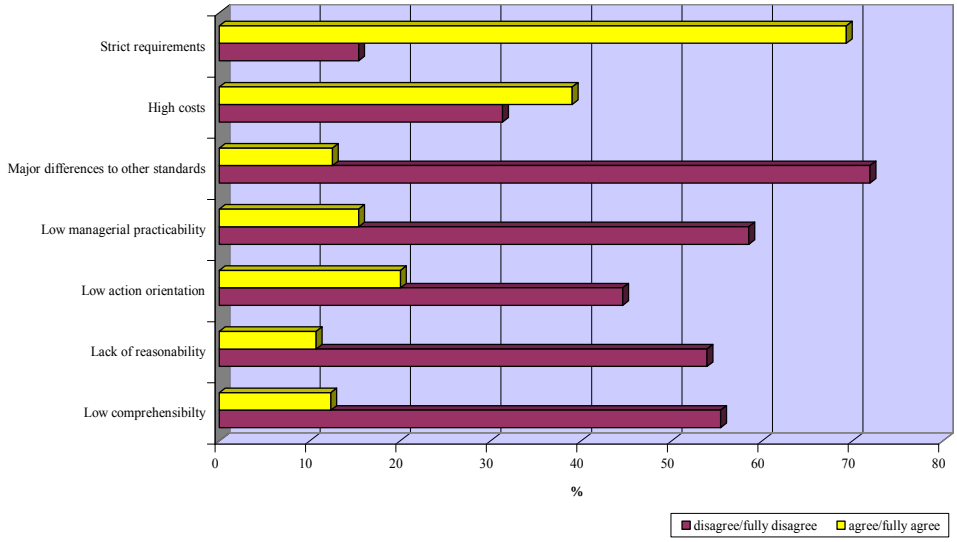

Fig. 4. Perceived disadvantages of the IFS [Gawron & Theuvsen, 2009a]

Taking the statements about the advantages and disadvantages of the IFS as a basis, a hierarchical cluster analysis was conducted. After the elimination of two outliers by using a dendrogram and applying the ward method, three clusters were identified by comparing mean values Gawron & Theuvsen, 2009a]:

- **Cluster 1: "The managers" (n = 23):** This firms generally have positive attitudes towards the IFS regarding internal effects such as the initiation of a continuous improvement process, the improvement of employees' quality motivation and positive influences on internal and external business processes. The companies in this cluster expect a reduction in the number of audits. Most members of this cluster are medium-sized companies with one to five hundred employees, except for nine companies, which are large scale manufacturers with more than four production sites and more than three thousand employees. The share of private labels produced varies remarkably (between less than and 25% and more than 75%). This cluster includes producers of dairy, frozen, meat and convenience products.
- **Cluster 2: "The supporters" (n = 27):** The firms in this cluster predominantly stress that the IFS requirements are well structured; they perceive a high action orientation and high managerial practicability. Statements concerning a lack of reasonability and comprehensibility are strongly rejected. Reductions in the number of audits and in audit and certification costs are not expected. "The supporters" perceive more advantages than disadvantages, but, unlike the members of the first cluster, they do not use the IFS as a managerial tool that helps to improve business processes and employee motivation. The cluster mainly consists of small companies with less than five hundred employees

and not more than three manufacturing sites. The percentage of private labels produced is below 50% in all companies. The cluster includes producers of bakery products, meat processors and confectionary and snacks producers.

- **Cluster 3: "The rejecters" (n = 13):** The companies in this cluster have been certified although they perceive neither cost reductions nor any other advantages. The respondents were disappointed by the standard structure. All in all, this critical group is very heterogeneous with regard to company size and product spectrum.

The results of the cluster analysis were tested with discriminant analysis. The results showed that 92.1 percent of the objects in the identified clusters had been classified correctly.

The study referred to above has been significantly extended by Schulze et al., (2008) by surveying all firms which in February 2006 were certified according to the 4th version of the IFS (1,799 companies). A total of 389 online questionnaires were returned (return rate: 21.6%). Respondents were mainly located in Germany (55.0%), France (9.3%), Italy (6.9%) and Austria (6.4%). On average, 346 employees work in the companies. The majority belongs to the following industries: beverages (20.7%), agricultural/horticultural produce (16.1%), meat products (13.2%), dried goods (12.9%) and dairy products (12.1%).

74.6% of the companies are generally satisfied with the IFS; however, only 32.7% would have implemented the IFS in the absence of any retailer requirements. This parallels findings with regard to traceability systems in the food industry; investments in these systems are also strongly dependent on external pressures, mainly from big retailers [Hollmann-Hespos, 2008]. 70.6% of the respondents emphasize that the advantages of the IFS outweigh its disadvantages. 82.2% perceive some useful input for operational management and 51.3% expect improved relationships with customers. 56.2% of the companies agree that the IFS has essentially contributed to increasing food safety and for 64.3% the IFS improved the food safety management. With regard to the costs of the IFS, the amount of time which the companies spent for the certification process is the most important aspect. For only 43.0% of the corporations are the operational expenses well justified by the benefits (cost-benefit ratio). 44.8% of the companies had to hire additional staff for the certification process.

In a second step, 25 theoretically derived statements about potential strengths and weaknesses of the IFS that were used in the questionnaire were reduced by means of a factor analysis. After minor modifications for double loading and non-loading items, the measures demonstrated acceptable levels of fit and reliability (KMO = 0.826; explained variance = 63.5%). All constructs revealed reliability, i. e. an α greater than 0.70 [Nunnally & Bernstein, 1994]. Only the two constructs "perceived costs of the certification" (0.54) and "perceived effectiveness of the auditor" (0.57) have lower reliability scores. Nonetheless, these constructs were not eliminated since they can be well justified from a theoretical perspective. All in all, six factors representing respondents' perceptions of the IFS were extracted (Table 1).

Table 1

Factors representing certified firms' perception of the IFS [Schulze et al., 2008]

Construct	Mean	Standard deviation	Factor loading
Perceived cost/benefit ratio[1], Cronbach's alpha = 0.883			
Would you agree that the IFS has improved your food safety management?	0.97	1.39	0.804
The administrative effort is well justified by the benefits from the IFS.	0.22	1.41	0.753
The IFS has essentially contributed to increasing the safety of our food production.	0.63	1.56	0.747
The IFS makes our business processes more transparent.	0.29	1.58	0.742
The advantages of the IFS outweigh any disadvantages.	1.10	1.39	0.725
Would you agree that the IFS has improved your relation with your customer?	0.49	1.59	0.677
The operational expenses of implementing the IFS are justified by its benefits.	0.34	1.38	0.666
Implementation of the IFS provides us with some useful input for our operational management.	1.64	1.11	0.579
Perceived quality of the IFS compared to other quality assurance systems[1], Cronbach's alpha = 0.864			
Compared with other certification standards, the IFS is more transparent.	0.26	1.48	0.891
Compared with other certification standards, the IFS is more efficient.	0.21	1.42	0.871
Compared with other certification standards, the IFS is more relevant.	0.26	1.48	0.807
Compared with other certification standards, the IFS is less bureaucratic.	-0.10	1.61	0.759
Perceived communication of the standard owner[2], Cronbach's alpha = 0.862			
With the information provided by the IFS offices, I am…	0.35	1.14	0.899
With the communication with regard to the IFS offices I am ...	0.48	1.17	0.862
With the information available on the Internet I am …	0.57	1.02	0.772
Regarding the information on IFS, I am …	0.76	0.95	0.701
Perceived expertise of the auditor[1], Cronbach's alpha = 0.708			
What was your level of satisfaction with the work performed by the auditor during your most recent audit?	1.64	1.02	0.796
The IFS auditor's expertise was absolutely adequate.	1.59	1.21	0.754
The IFS auditor had really no clue about the evaluation.	-2.02	1.11	-0.691
The IFS auditor, informally, made a number of useful suggestions.	1.44	1.32	0.584
Perceived costs of the certification[1], Cronbach's alpha = 0.538			
We had to provide additional staff for the IFS certification process.	0.16	1.95	0.790
The amount of time which we have to spend on the certification process is quite high.	1.63	1.29	0.789
Perceived effectiveness of the auditor[1], Cronbach's alpha = 0.565			
'Black sheep' will be singled out by the audit.	0.82	1.51	0.743
The IFS auditor really tried to point out weaknesses.	1.56	1.19	0.657
The IFS auditor was very exact.	1.95	0.94	0.521

KMO = 0.826; explained variance = 63.50%; [1] = Scale from +3 = fully agree to -3 = fully disagree; [2] = scale from +3 = fully satisfied to -3 = fully dissatisfied

Schulze et al. (2008) measured the impact of the factors identified (using factor scores of the extracted constructs and further selected variables) on the overall evaluation of the IFS by applying a stepwise least squares model using the method of ordinary least squares (OLS) as the estimation procedure. The model was highly significant (F-value = 80.72) and 54% (= adj. R square; R = 0.74) of the overall evaluation are explained by the regression equation (Table 2).

Table 2

Overall evaluation of the IFS [Schulze et al., 2008]

Independent Variables	Coefficient	Std. Error	Beta value	T value
c	0.859	0.040		21.245***
Perceived cost/benefit ratio[1]	0.346	0.038	0.396	9.052***
Evaluation of the IFS catalogue of requirements[2]	0.275	0.043	0.294	6.380***
Perceived communication of the standard owner[1]	0.203	0.033	0.235	6.181***
Perceived expertise of the auditor[1]	0.195	0.032	0.227	6.173***
Perceived costs of the certification[1]	-0.099	0.032	-0.117	-3.077**

Dependent variable = "overall evaluation of the IFS"[3]; F-value = 80.72***; Std. Error = 0.586; adj. R^2 = 0.54; R = 0.74; *** = p<0.001, ** = p<0.01, * = p<0.05; c = constant; [1] = factor values; [2] = scale from +3 = fully agree to -3 = fully disagree; [3] = scale from +3 = fully satisfied to -3 = fully dissatisfied; independent variables (not significant): "perceived quality of the IFS compared to other quality assurance systems"[1], "perceived effectiveness of the auditor"[1], "perceived external pressure" (= We would have implemented the IFS even in the absence of any retailer requirements)[2], "country" (= Germany, France), "number of other quality assurance systems" (= BRC, ISO 9001), "number of employees", "member of a larger production group".

The results show that certification costs are not as relevant as expected earlier. Instead, the perceived cost/benefit ratio turned out to be the most important determinant of a firm's assessment of the IFS. Furthermore, the evaluation of the companies of the IFS catalogue of requirements, the communication and information of the standard owner and the perceived expertise of the auditor are more important for the evaluation of certification standard than perceived costs of the certification.

A subsequent cluster analysis by and large confirmed the results of the much smaller earlier study by Gawron and Theuvsen [2009a]. Again, three clusters could be identified. "The unconcerned" (29.1%) are generally satisfied with the IFS but do not perceive many advantages. Most members of this cluster are larger companies with about 450 employees on average. The second cluster "The satisfied" consists of 40.7% of the companies surveyed. This cluster manly consists of small companies. All in all, they have a very positive attitude towards the IFS regarding internal (for instance, useful inputs for the operational management) and external (for instance, an improvement in relationships with costumers) effects. However, these companies perceive high costs during the certification process. "The dissatisfied" are medium-sized companies with on average 311 employees. The members of this cluster do not see any positive effects on food safety or on their business relationships with customers. They found the implementation of the IFS difficult and perceive high costs during the implementation phase and a negative cost/benefit ratio [Schulze et al., 2008].

Discussion and Conclusions

Today certification systems play various roles in food supply chains. By and large, these roles can be summarized under the key word 'reducing information asymmetries in agri-food chains'. Due to their fast diffusion, certification systems have become a *conditio sine qua non* for supplying to large retailers. Therefore, these systems nowadays can, at best, be classified as quasi-voluntary [Meuwissen et al., 2003]. Against this background it is not a surprise that certification systems have also gained much relevance in many Central and Eastern European countries especially if these countries extensively deliver into Western European agri-food chains.

The empirical results show that the overall evaluation of the IFS is positive but that there are also a considerable number of firms that still have negative attitudes towards certification systems and do not see any advantages of implementing them. The regression analysis reveals that the evaluation of the IFS is more positive if food manufacturers perceive a good cost/benefit ratio of certification procedures. It also shows that firms are not only interested in receiving the certificate but mainly in food safety benefits. Furthermore, some respondents report positive effects on their companies, such as continuous improvement processes or improved quality motivation of employees. This has important managerial implications for standard owners since it is obviously very important to meet firms' expectations with regard to the benefits of certification systems. Since the catalogue of requirements offers the most important opportunity for improvement, the standard setter has a chance to enhance the satisfaction of certified companies. This could be achieved by the integration of more subsector-specific requirements and the benchmarking of the IFS against other certification standards. It may also be helpful to better communicate the advantages of certification systems through, for instance, regular newsletters, an earlier announcement of upcoming changes, or provision of more industry specific information and suggestions for successfully implementing standards.

The studies summarized above also have managerial implications for IFS certified firms. Companies that do not yet perceive any advantages with regard to the implementation of the IFS should consider the standard more as a quality management instrument. Some companies already observe improved internal business processes. These companies can serve as benchmarks for the more reluctant and sceptical food processors [Gawron & Theuvsen, 2009a; Schulze et al., 2008].

Future research should take a closer look at other standards, especially those that also address farms. It will be interesting to see how the "farmographics" [Fernandez-Cornejo & McBride, 2002] such as farm size, farmers' age and formal training affect the assessment of certification systems. Future studies can also analyze in more detail how certification systems can be designed in order to make them more attractive for farms and firms and increase owners' and managers' intrinsic motivation to implement these systems and comply with the standards set. Starting points are provided, for instance, by the modern theory of bureaucracy that aims at distinguishing between "good" (enabling) and "bad" (coercive) bureaucracies [Adler & Borys, 1996; Adler, 1999]. Theuvsen (2005) has suggested to increase the flexibility of standards and to enhance participation of certified farms and firms in the process of designing certification systems.

Only limited knowledge exists so far about the influence of prior experiences with food safety incidents and product recalls on the assessment of certification systems. Findings

with regard to investments into tracking and tracing systems indicate that these incidents may have an influence [Hollmann-Hespos, 2008]. Similar observations were made with regard to the use of animal health-related information by farmers obtained from slaughterhouses [Deimel et al., 2008]. Nonetheless, more systematic research with regard to contingency factors influencing farmers' and firm managers' perceptions with regard to certification systems is needed. These studies should also include cultural aspects that have hardly taken into account so far.

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2

TRACEABILITY SYSTEM AS A CRUCIAL PRODUCT SAFETY FACTOR IN THE FOOD CHAIN

Introduction

Traceability is the possibility to follow the origin and location, and recognise a unit product or a batch of products at each stage of production, processing and distribution [Czarnecki, 2005; Kijowski & Nowak, 2006]. Its aim is to identify the path which a product or a lot of products has followed at all stages of the production chain and the supply chain [Pugh, 1973; Moe, 1998]

Traceability of products may be realized in two directions: downward (tracking) or upward (tracing). Tracking is used to identify e.g. the producer, supplier or lot number of a lot of raw material in order to determine the cause and source of hazard. In turn, tracing makes it possible to specify the site to which the defective lot of products was delivered [Kijowski & Cegielska-Radziejewska, 2008; Stein, 1990].

The traceability system facilitates prompt identification of potentially harmful products in the production chain and their withdrawal from retail, thanks to which products offered by a given plant are safe for the consumer.

General principles and basic requirements concerning the design and implementation of the traceability system are given in the standard PN-EN ISO 22005:2007: *Traceability in the feed and food chain - General principles and basic requirements for system design and implementation.* [PN-EN ISO 22005:2007].

The structure of traceability system

The structure of the traceability system includes four elements (Fig. 1): organization, process, information and technology.

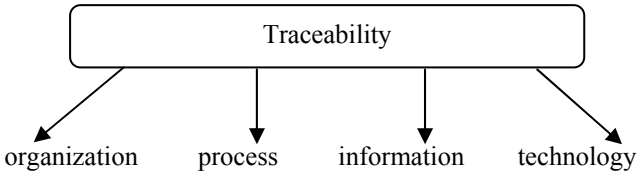

Fig. 1. The structure of traceability system

When implementing the system it is necessary to define the components of the traceability system in the following way [Smith & Furness, 2006]:

a) organization – all members of the food chain need to be identified and the scope of their responsibility for the final quality of the product needs to be determined,
b) process – individual stages of production and distribution processes need to be determined for a given product and requirements concerning record keeping and monitoring at each of these stages need to be specified,
c) information – it is necessary to determine what information concerning the product should be followed throughout the entire food chain. This makes it possible to realise the goals of the enterprise in relation to production of safe, superior quality products,
d) technology – it needs to be specified what technology and what tools are used during production.

The traceability system guarantees the flow of information over the entire food chain. It includes in its scope the origin of materials, history of processing and distribution of analyzed foodstuffs [Derrick & Dillon, 2004]. Figure 2 presents an example of a model of traceability system applied in food industry with indicated directions of information flow along the food chain [Zadernowski & Obiedziński, 2005]:

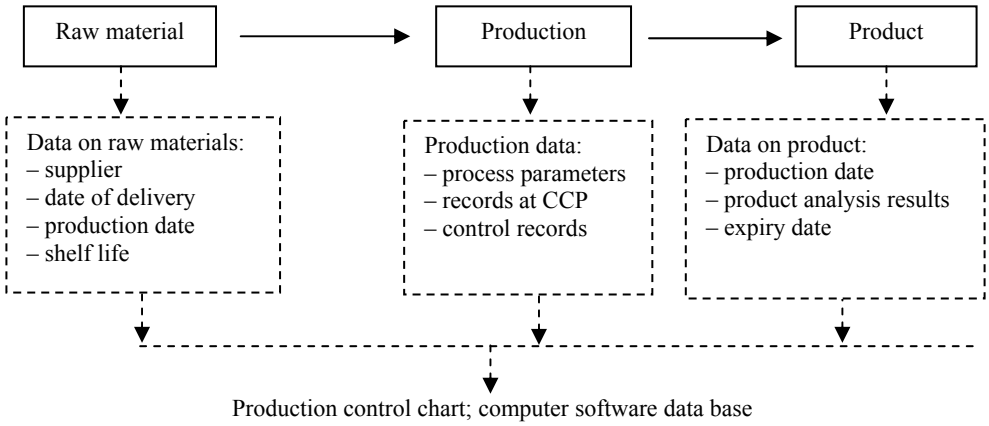

Fig. 2. An example of a traceability system model

There are two levels for the implementation of traceability system: the internal traceability system and the supply chain traceability system. The first level is connected with the flow of information concerning raw materials, semi-finished products and final products only inside the plant, while the other – with information flow throughout the entire food chain and concerns all organizations belonging to this chain [Kijowski & Cegielska-Radziejewska, 2008; Sarig, 2003].

Material and methods

Experimental material consisted of "Herring fillets in oil", which were collected from a warehouse of final products of a fish processing plant, where these investigations were carried out.

The aim of the study was to verify the traceability system operating in the analyzed fish processing plant. This verification included traceability of "Herring fillets in oil" according to the requirements of traceability system, specified in the standard PN-EN ISO 22005:2007 *Traceability in the feed and food chain: General principles and basic requirements for system design and implementation*. Traceability consisted in tracking "Herring fillets in oil" in the plant from the final product to the raw material, i.e. from the production planning department, through the department of fish raw material acceptance, the laboratory, the department of raw material preparation, the production department, up to the warehouse of final products. Product tracking was based first of all on documents found at individual departments of the fish processing plant.

The course of analyses

The path of "Herring fillets in oil" was followed according to the scheme of tracking of fish products from the final product to the raw material (Fig. 3).

Tracking was started at the production planning department, where a production order from the last production of "Herring fillets in oil" was collected, on the basis of which the following data were determined: production date of this product, expiry date, product number and fish lot number. The next stage in the analysis was to find a respective fish raw material acceptance protocol at the department of fish raw material acceptance, based on the known fish lot number. Among other things, the delivery date of the fish raw material and the name of its supplier were read from the protocol. Next at the laboratory sensory examination protocols of "Herring fillets in oil" were collected based on the production order number. A list of raw materials comprising the analyzed product was prepared on the basis of data obtained from the department of raw material preparation. Next documentation found at the production department was reviewed concerning weight control, detection of heavy metals in the final product and measurement of product temperature. Then based on the production order number a list of production orders for "Herring fillets in oil" was collected from the warehouse of final products based on the number of production order, which in turn was used to determine the consignee of the product, the date of its shipment and the ordered quantity of the product.

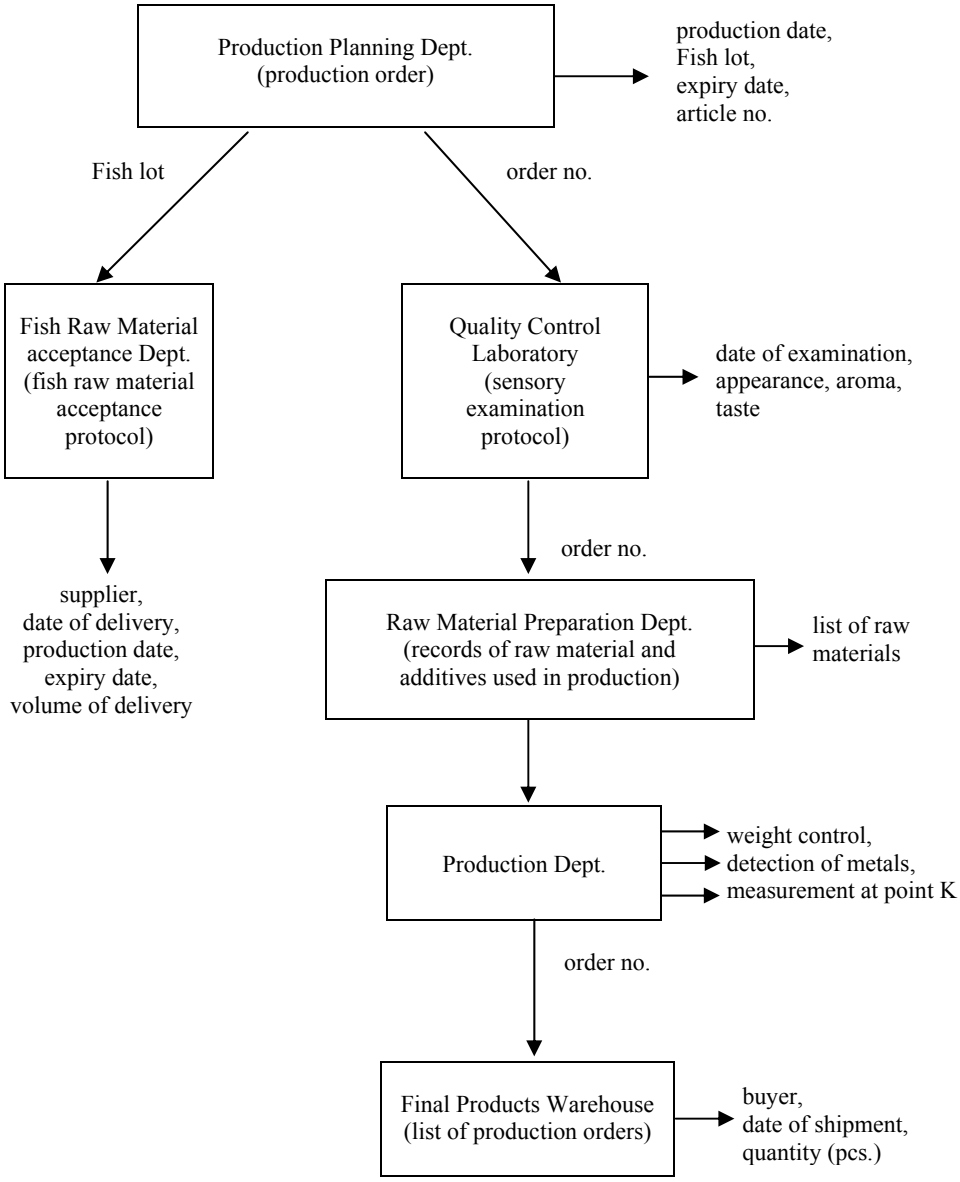

Fig. 3. The scheme of tracking of fish products from the final product to the raw material

Results

Traceability results of "Herring fillets in oil" are presented in Tables 1–3.

Table 1 lists basic information concerning "Herring fillets in oil", such as production date, the quantity of the produced product and its expiry date. Moreover, lot number of fish raw material used in the production of "Herring fillets in oil" was also given, as this information is required in case it is shown that the defect of the product was caused by the use of raw material of inadequate quality. Table 1 contains also data concerning sensory examination of "Herring fillets in oil" (point 2), results of weight control (point 5), the presence of metals in the final product (point 6) and measurement of product temperature (point 7). On the basis of such information it was possible to identify the possible cause of a defect in the final product. Table 2 lists raw materials comprising "Herring fillets in oil", together with their delivery dates, suppliers, the volume of delivery, production date and expiry date. These data may be helpful assuming that any of the used raw materials resulted in a poor quality product. In turn, Table 3 gives consignees of "Herring fillets in oil" together with the ordered quantity and the date of shipment of this product. Such information is required in case when it is necessary to withdraw the entire lot of the product from the market – then it is necessary to determine all suppliers of this product.

Table 1

Herring fillets in oil

1. Basic data	
Product No.: 17300	Fish lot: 4200
Production date: 23.05.2006	Production order: 805211
Expiry date: 24.07.2006	Produced quantity: 950 pcs
2. Sensory examination report of final product	
– evaluation date: 24.05.2006	– appearance, aroma, taste, consistency of product – appropriate
3. List of raw materials	
Raw materials for the production of "Herring fillets in oil" are presented in table 2	
4. Description of fish material	
– fish lot: 4200	– supplier: Nord Capital
– delivery date: 07.05.2006	– expiry date: 10.06.2006
– volume of delivery: 17000 kg	– temperature of supplied raw material: – 15^{o}C
5. Product weight control	
– date: 23.05.2006	
– required product weight: 200 g	– actual product weight: 201; 203; 204; 203; 200; 202; 203; 201 g
6. Detection of heavy metals in final product	
– date: 23.05.2006, time: 4 p.m.	– no heavy metals detected in final product
7. Measurement of product temperature	
– date: 23.05.2006	– temperature: 15.2^{o}C
8. Release of product no. 17300 and expiry date 24.07.2006.	
Distribution of "Herring fillets in oil" with expiry date 24.07.2006 is presented in Table 3.	

Table 2

Raw materials for the production of " Herring fillets in oil"

Raw material	Date of delivery	Production date	Expiry date	Supplier	Volume of delivery
Edible oil	22.05.06	-----	-----	Milo	-----
Salt	10.03.06	12.01.06	12.01.09	ESCO	
Vinegar 80%	17.02.06	16.02.06	16.02.07	Nowichem	4000kg
Sodium benzoate E 211	04.01.06	08.09.05	08.09.07	Supero	700kg
Citric acid	18.04.06	10.02.06	01.2008	Supero	-----

Table 3

Distribution of "Herring fillets in oil" product no. 17300

Ordered quantity	Date of shipment	Consignee
150 pcs	26.05.2006	Real Kraków
70 pcs	26.05.2006	Market Piotr i Paweł Warszawa
100 pcs	26.05.2006	TESCO Warszawa
50 pcs	26.05.2006	Żabka Oddział Poznań
80 pcs	26.05.2006	Biedronka Wałbrzych
100 pcs	26.05.2006	Auchan Poznań
50 pcs	26.05.2006	Real Warszawa
200 pcs	26.05.2006	BIG FISH Katowice
150 pcs	26.05.2006	TESCO Poznań

Concluding remarks

It was found that based on the data concerning "Herring fillets in oil", obtained from the documentation found at individual departments of the plant and the scheme of tracking of products from the final product to the raw material, it is possible to recreate the history of this product in the food chain. In case of possible irregularities in the production process, on the basis of obtained results it is possible to identify the stage at which they appeared, as well as find the cause of the possible defect in the final product.

In the analyzed plant product traceability is possible thanks to the maintenance of continuity in information flow over the entire production chain. Continuity is preserved as a result of knowledge of the production order number, since on this basis information may be obtained on "Herring fillets in oil" from successive departments of the plant.

Implementation of traceability system in a food processing plant makes it possible to offer products not only characterized by desirable quality parameters, but also safe for the consumer. Traceability system provides for information flow throughout the entire food chain, thus contributing to increased assurance of food safety. When implementing traceability system it is necessary to determine the goal which a given organization intends to achieve by implementing this system. At present traceability system is most frequently implemented in order to guarantee consumers than the product they buy is completely safe and exhibits desirable quality parameters, thus increasing competitiveness of enterprises on the food market.

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3

THE ASSURANCE OF QUALITY AND SAFETY OF FOOD PRODUCTION ON THE EXAMPLE OF MEAT AND MEAT PRODUCTS

Introduction

Consumer safety has become a priority task in food chain management. Despite the efforts made by participants in the food chain, it is absolutely impossible to eliminate the risk connected with the food safety hazard. That is why all operators of the food chain supply on all production stages, processing and the distribution inside companies controlled by them, should assure, that the food quality meets the sanitary-hygienic requirements [ECR Europe, 2004].

For this purpose companies of food branch must possess the effective system of identification for own suppliers of food products or fodder, breeding animals and other different substances, which could be the component of final food products. The identification of risk sources by traceability system allows to reduce the range of potential occurrence of the hazard among the other participants of food supply chain. This fact allows to eliminate the risk of supplying hazardous food which could be harmful for consumers' health.

The duty of the monitoring circulation and the origin of food and fodder (traceability) for the purpose of food safety supplied to the European Union, also in Poland, is a direct result from the Regulation (EC) No 882/2004 of the European Parliament and of the Council from the 29th April 2004. Introduction and using the traceability system has been a legal requirement since the January 2006 [Regulation (EC) No 853/2004 and No 854/2004].

The aim of this paper was to present of assurance quality and food safety production system on the example of the chosen meat processing plants, with special regard to functioning of the traceability system of meat and their products.

Traceability

The essence of traceability is a possibility to track and trace the origin of given products (production lot) on every stage of supply chain. The identification gives the possibility to obtain data from the previous stage of the chain (from who and what received?), next delivery of information to the next stage (to who and what sent?) [ECR Europe, 2004; Schwägele, 2005; Lech & Przybylski, 2007]. The flow of the stream of information was presented on Figure 1.

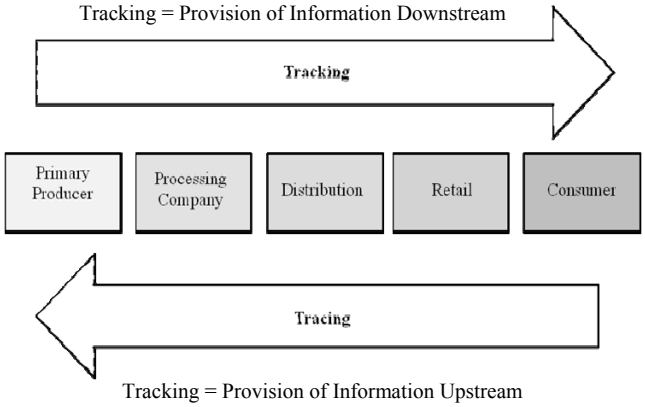

Fig. 1. Tracking and tracing along the food chain
Source: Schwägele, 2005

Following the movement (so-called Tracking) allows to determine the actual status of shipping, however following the origin (so-called Tracing) helps to obtain information enabling to reconstruct the history of shipping [Rules of tracking ….., 2007].

Traceability in the reference to the meat

In the meat industry product quality is understood as all quality aspects, important for the consumer 'from farm to fork'. They are: food safety, animal welfare, environment, healthiness, flavour, lifestyle [Wood et al., 1998].

Contemporary integrated quality systems make it possible to follow the whole history of the productive process of meat and meat products. All information of animal breeding and meat processing after slaughter topic, starting from the farmer (breeder), and finished on the final consumer can make it accessible (Figure 2). Every partner of the system is registered and possesses the individual information number for identification. This system works in the range of Feed-Farm-Processing-Assurance.

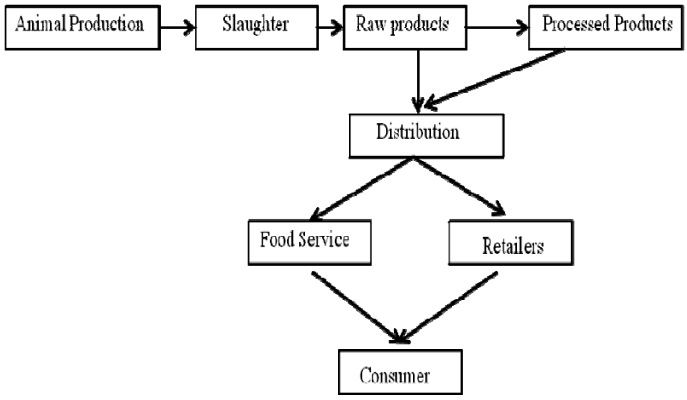

Fig. 2. Farm to Fork food safety
Source: Sperber, 2005

Functioning of traceability in the reference to the meat on McDonald's Corporation example

McDonald's Agriculture Assurance Programme (MAAP) is the own system of the quality assurance, created and implemented in 2003 because of McDonald's corporation needs. This system was based on international quality systems in industry and agriculture. Standards for the meat are based among other on the popular in the meat industry German quality management system Q & S (Qualität Und Sicherheit), which consists of production, processing as well as logistics. Standards which semi-manufactured articles supplier must fulfill are described in detail in MAAP system. Standards concerning the various areas were presented on Figure 3. One standard can concern several problems.

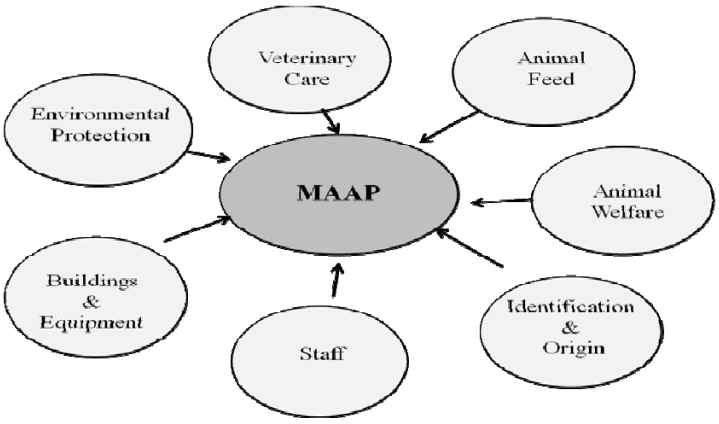

Fig. 3. MAAP areas from the range of animal materials production

The Environmental Protection Area contains problems concerning among others things: the air, soil, water and waste management. For example, producers should obey the following rules of Good Agricultural Practice, understood as the level of farmers' competence as for breeding and cultivation. The applied cultivation methods and farming techniques should not cause permanent damage to the environment. For this purpose various preventive actions are undertaken. Every plant/ production unit must possess the written Waste Management Plan and the map on which the areas of the increased risk were marked. The plan informs about the ways of the potential hazards problem solving, e.g. removing carcasses, used containers, elements made from plastic, used disinfectants, out of data/ used medicines, used needles and syringes etc., or storage of fuels, disinfectants, medicines and others. Moreover, standards in this area also concern the minimization of waste, and the optimization of energy use.

Buildings & Equipment Area contains detailed issue concerning the construction and the maintenance of the buildings (e.g. the renovation) and assurance of proper conditions for animals (space norms, ventilation, lighting, air temperature), waste storage, and also transporting facilities.

The Staff Area contains issue relating to Health & Safety issue, staff trainings, possession of emergency action plans. Workers are trained among others about the principles

of stressless and not hazardous for health and life hazard dealing with animals. Knowledge of the slaughter animals nourishment, and also of animals behaviors (skill of the recognition of basic disease symptoms, stress situation) is verified.

Identification & Origin Area contains aspects allowing animals' identification. This mainly relates to marking by earrings and cattle passports. It allows to identify the animal from the place of birth to the last breeder and also to prepare detailed documentation: book and herd trade [Regulation (EC) No 1760/2000; Dz.U. 2007, No 52, 345].

Nourishment Animals Area covers the fodder problems. The fodders which animals are feed must be appropriately selected, and come from the audited feed mills. All components of fodders must be obligatorily free from GMO (Genetically Modified) and from any contamination. Applying any growth stimulants and animal originated proteins is forbidden in the animal nourishment. Using antibiotics could be only considered when the necessity of animals treatment occurs and then the appropriately long waiting period is needed.

In Animal Welfare Area there is a standard of the humanitarian practices with animals, supervision and the observation of their behaviours. Assuring proper care, supervision, and living conditions of the animals, which means freedom from hunger and thirst, freedom from discomfort, pain, injuries and diseases and freedom to express normal behaviour (monitoring of animal behaviour).

In case of cattle factors such as: surface of stable per 1 head of cattle, suitable ratio of window surface to floor surface, temperature, lighting in the stable, air flow (air humidity, the concentration of harmful gases), ventilation, appropriate bedding (enabling free moving and not exposing the animal to falls), proper nourishment and general animal treatment are taken into consideration.

In case of chicken, requirements related to slaughter birds breeding concern: the way of breeding (e.g. barn egg system, whole number of birds, stocking density, free access to the perch, to bedding and to drinking water, the parameters of ventilation, air flow, temperature, permissible concentration of harmful gases, air humidity, suitable lighting, ratio of windows surface to floor surface). Moreover, the birds should not be exposed to stress and any other inconveniences. In the European Union a lot of attention has been drawn to animal welfare. The Union legislation contains a number of legal acts of animal welfare improvement published in the last years [Regulation (EC) No 882/2004]. Currently the requirements concern new and rebuilt buildings, but starting from 1th January 2013 they will be obligatory for all producers. In Poland the Regulation of Ministry of Agriculture and Rural Development i*n the matter of maintenance of minimum conditions for individual species farm animals* [Dz. U. 2003, No 167, 1629] is also be in force. Veterinary Care is the next area of the MAAP system. At the farm a veterinary care should be assured. It must contain a plan and documentation of animal veterinary treatment, the applied pharmaceuticas (antibiotics with withdraw time save for animal treated by pharmaceutical) and ill animals` isolation.

Every semi-finished product supplier for McDonald's restaurants must assess the degree of fulfilling the MAAP standards. The first stage is fulfilling the MAAP Supplier Self Assessment Form. The compliance with the standards is assessed by the following scale:

- Full Compliance if all requirements are met,
- No Compliance if no requirements are met,
- Partial Compliance if some requirements are met,
- Not concerning, in the case of other products.

The degree of the compliance with the MAAP system is expressed as the 'compliance coefficient' obtained by analysis of the requirements of the given standard and estimating the percentage of the compliance.

Supplier must also define how he is able to verify Full or Partial Compliance, e.g. by audits conducted by the external firm, by national inspection and obligatory (national legislation) or by own internal assurance quality system covering the standards required by McDonald's.

In case of poultry, the whole process of meat production is supervised by the MAAP system. Starting from feed mill components of mixtures and proportion of their addition are controlled. The next hatchery of parents flock, parents flock, hatchery broiler, and poultry keeping are controlled. Every stage of production contains the separate standard of the MAAP system. Poultry production achieves very high compliance coefficients with the MAAP system, because it is possible to carry out the full identification of raw material coming from chicken farms. There is also a possibility to trace all factors significant for the quality, even reaching to grand-parental generation breeding slaughter birds.

In case of cattle the controlling system because of it is not so advanced considerable farms disintegration and limited contact with breeders, resulting from the fact that beef livestock turnover is done mainly by agents. However many of cattle breeders fulfill these standards to a large extent. Cattle identification in the UE consists of the following elements: ear tags to identify animals individually, computer databases, animal passports, individual registers kept at each farm [Regulation (EC) No 1760/2000, Dz. U. 2007, No 52, 345]. In the cattle passport there is the following information: animal identification number, number of the seat of the herd, name, surname (business name) and address of the animal keeper, date and place of the animal birth, sex, breed, identification number of the animal mother, the origin of the animal, date of slaughter or death of the animal.

Additionally every animal possesses an ear tag with the following information: unique identification code of the animal, bar code and the country symbol. In case of McDonald's corporation, procedures of beef carcasses elements potential contaminated by BSE (Bovine Spongiform Encephalopathy) prions are implemented. For this purpose, the contaminated elements are dyed blue on the production line and rejected from processing. Twice a year, GMP audit including SRM (Specified Risk Material) audit, takes place in the plant for effectiveness assessment of the procedure.

After receiving the raw meat material, it is controlled and Traceability Form – special form of raw material acceptance – is prepared. The acceptance of the incoming raw meat material (poultry and beef) to the plant is a Critical Control Point (CCP 1) of HACCP system. Meat elements subjected to the control are presented in Table 1. To avoid any mistakes and cross-contamination, the containers designed for poultry transport are of yellow colour, whereas containers for beef transport are blue.

Table 1

Parameters controlled during acceptance procedure of raw meat delivery

No.	Poultry raw meat material	Beef raw meat material
1	Transporting data: conditions of transport facilities for raw meat delivery. Driver certificate for competence to meat transport.	
2	Documentation of origin, transporting conditions and raw meat quality specification	
3	Proper containers labeling system (number of raw meat element, information about supplier, date of slaughter and date of arrival of raw meat).	
4	Address and farm details. Farm code.	Date of animals slaughter
5	Address of feed mill which prepare raw material for feed. Feed mill code.	Date of carcasses cutting.
6	Data of parents birds farm. Name and localization of feed mill of parents birds.	Date of freezing (if meat is frozen).
7		Raw meat material class
8		Negatively result of BSE examination*.
9		Stamp control (brown stamp - safe, green stamp- cut out from carcasses).
10		Putting down meat elements of individual animals to specified containers (on the basis of ear tags), and separation of meat elements to lean and fatty class.
11	Meat net weight (gross weight). Meat temperature. Measurement of skin and tendon content, monitoring the presence of foreign bodies (glass, plastic etc.), monitoring the presence of haematomas, thrombuses, contents of bone crumbs, untypical colour. Evaluation of freshness (aroma, colour).	

* meat exclusively from beef fronts is used for processing

Source: unpublished McDonald's data, 2008

After receiving the raw meat material in the plant, is stored in the cooler. Storage temperature is the critical parameter (CCP 2). Preparing of freezing meat semi-finished products (poultry, beef) is the next step of the process. These products are used later in McDonald's restaurants. Firstly, in a plant, poultry semi-finished products are subjected to heat treatment and temperature inside product is Critical Control Point (CCP3). It should be pointed that all poultry products in McDonald's are HT (heat treatment). Ready frozen meat semi-finished products are individually packed and after packaging are checked by metal detector on the line. This step is Critical Control Point – CCP4. Individual packages are packed in labeled cardboard boxes and stored in the cooler. The storage temperature is the next critical parameter – CCP 5. Final products are tested by qualitative tests during the processing. The products are prepared in the same conditions of heat treatment as in the restaurants and then checked if they fulfill specified quality standards. On the basis of special forms, sensory analysis and physical parameters (weight, diameter, thickness etc.) are assessed.

Moreover, Traceability Test is regularly conducted. The test enables the most effective way of finding the hazards origin by tracing the history of products from the restaurant to the raw meat material producer.

According to Global System GS1, to achieve the best effectiveness of tracing the consumer and logistics units are applied. Effectiveness of tracing is obtained by using bar codes of GS1 -128 type. It allows to code logistics' information in one bar code. Identification standards allow quick and faultless localization of the products and removing the defective ones from the market, if necessary. In the critical situation, the corrective action is realized immediately and accurately with faultless localization of suspected elements of the product and current place of storage [Narzędzia do zarządzania "traceability"…., 2004].

At the meat plant all accepted lots of raw meat materials are marked by specific labeling system. Similarly, every lot of the final product is labeled. Individual cardboard box possess its own identification code which contains information about the identification processing term and the identification of raw meat material used for processing. During processing every step is monitored. The elements of raw meat material in every lot could be identified in each step of the processing line. The system enables to remove each element which does not fulfill quality requirements and these ones which could have had recognized the defective ones.

Summing up, the system gives repeatable, standards quality of final products and enables McDonald's corporation to achieve steady quality requirements in all restaurants. Moreover, quality and safety requirements of the European Union are fulfilled as well.

Assessment of traceability system effectiveness by the example of red meat processing plant

Traceability system correctness was assessed by the study of the production chain of the beef (beef leg) and pork (steamed cracow sausage) products of the largest and the most modern meat plant in Poland. The producer has a high position among the best plants because of modern technology implementation, systematic modernization and the experienced and qualified staff.

Every head of the slaughtered livestock is examined by independent state veterinary inspection. The quality of the received raw meat material and meat final products is analyzed by the plant laboratory as well. Everyday plant laboratory conducts the analysis of microbiological cleanness, chemical, physic-chemical properties and sensory analysis. Every stage of processing is realized in conformity to Good Hygiene Practice starting from requirements of receiving livestock, through all processing steps up to storage and preparation of transport lots for customers. Slaughtered heads of cattle above 24 months old are systematically examined for the BSE prions presence. HACCP system is implemented and verified which guaranties safety at every step of processing. The plant posses export licenses to the European Union market, Russia and 15 other countries. The plant possesses a well developed own products distribution net. The raw meat material for processing is received from ecologically clean regions of Poland. The plant systematically builds the own raw material based on assuming the stable high-quality livestock.

The traceability system developed in the plant requires processing lots identification system in each processing department. The production Director is responsible for the supervision of implementation of all necessary procedures. Department managers are responsible for the supervision of procedures implementation and instructions in each department. The analysis of documentation related to the traceability system showed that the efficiency of the system is very high.

Traceability test – the case of beef quarter-carcasses with drawal from the market

In the discussed meat processing plant, traceability system is regularly (twice a year as minimum) tested. Verification of the traceability is realized from the reception of raw meat material to final product orders realization according to specification. The beef quarter-carcasses withdrawal from the market, simulated in crisis case, has documented procedure. The procedure of the critical situation of cysticercosis in several beef quarter-carcasses was analyzed. The complaint of cysticercosis in beef leg muscle was received 48 hours after animals` slaughter. Immediately after the official complaint, the crisis team was appointed and withdrawal from the market procedure was initiated. The defective lot of quarter--carcasses of 1678 kg withdrawal was completed within 56 hours from the processing in the plant (Table 2). The quantity and the buyers of the faulty meat were perfectly identified. The technological process and the supply documentation were analyzed precisely up to the place of animals` farming. The farm which delivered the questioned beef quarter-carcasses was established.

Moreover, it was established that inappropriately trained veterinary inspector labeled the faulty beef quarter-carcasses wrongly capable of retracing. He was relegated to additional training.

Table 2

The analysis of traceability system effectiveness of the meat plant

Product	Hazard	Corrective actions	Effectiveness	Time of realisation
Beef quarters carcasses	Complain of carcasses for measles presence	Crisis team appointment, and identification of: processing lot of 1678 kg of beef quarters carcasses; suppliers; distribution within departments; analysis of purchaser of whole beef quarters carcasses, complain card sent; withdrawal procedure starting; establishment of the hazard origin; undertaking the corrective action	Withdrawal of 100% of complained carcasses, 347 kg of beef processed products from complained meat identification. The whole lot of quarters-carcasses was investigated.	56 hours
Cracow sausage	Exceed the level of the salt content	Appointment of crisis team and identification of: processing lot in department of sausages production and in central storehouse, distributors and purchasers (retail shops), withdrawal procedure starting, analysis of hazard causes; undertaking the corrective action	Withdrawal of 64.79% of final product (lot of sausages was consumed)	55 hours

Source: own study

Traceability test in meat processing plant on the example of removal the Cracovian Steamed Sausage from the market

In case of the Steamed Cracovian Sausage was received a report from Laboratory informing about 4.1% of salt level in sausage whereas the permissible salt level should not exceed 3.0%. After receive this report The President of Trust was informed immediately. He decided to realize the Instruction of Product Removal from the market in the critical situation and to form a Crisis Team.

In the Central Storehouse the presence of faulty meal was not confirmed. Quantity and the directions of distribution introduced on the market of the faulty 'Cracovian Steamed Sausage' were identified. The list of purchasers informed about the product removal was enclosed. To all identified purchasers a Card informing about the product being removed from the market and agreed by phone on the way of receiving the faulty lot. The returned sausages were protected in allocated place in the Central Store house and labelled by a Stop tag.

The amount of the faulty lot of the production was 345.37 kg. The amount of 223.77 kg of sausage (64.79%) was removed from the market while 121.60 kg of Steamed Cracovian Sausage was not removed because of having sold it to the individual customers. The whole returned of lot was directed to utilization.

All productive stages were examined by the analysis of food safety of the objective part. Technological process of the production of the Cracovian Steamed Sausage was analysed. In particular quality of used spices, packaging and additions and the agreement of certificates with specification. In this matter no irregularities were confirmed. Expiry date of used spices was also checked and irregularities were not found as well.

Based on the qualitative and quantitative conformity of conducted production with the documents concerning the recipe was confirmed that the usage of raw material and the spices to the production according to the recipe and label was in full compliance with The Card Product. It was established which staff member was responsible for cutting meat by mechanical cutters and mixing. As a result of conversation with the employee it was established that by mistake he added 8 kg of edible salt which was placed, near spices prepared for Cracovian Steamed Sausage. By mistake salt was not with other spices in the Spices Store-house because of the correction of the order for Podwawelska Sausage. It was discovered that the worker carrying-out cutting meat was trained on mechanical cutters (record from Work Place Instruction), but the record from carry-out evaluation of the effectiveness of training was not found. In order to eliminate the cause of this discordance a training concerning all meat products productive processes to minimalize of the possibility of reoccurrence of similar situation. In all production departments additional training concerning the necessity of following the instructions at the place of work was conducted,

The action of removing faulty 'Cracovian Steamed' product - the removal of 65% of faulty product from the market – took 55 hours (Table 2). The completed analysis of efficiency of traceability system in the chosen meat processing plant showed that this system has a significant importance in the assurance of food safety.

Correctly implemented traceability system gives many possibilities to monitor the quality of products, but first of all it allows to remove rapidly and efficiently the article from the market in case of any hazards. In the discussed plant the simulation of product removal was conducted. It could determine the hazards for the consumer health or possibility of lack of compliance of the product with the requirements of the quality. This simulation unambigu-

ously showed that traceability system in this plant functions efficiently. It allows not only efficient removal of the product from the market, but also gives a possibility to recognize the causes of the hazards by the analysis of the product 'way' in the plant. The localization of the source of the mistake allows to avoid the reoccurrence of this mistake in the future, as well as to correct all actions leading to the occurrence of a similar problem.

Conclusion

The realization of traceability system in McDonald's corporation as well as the studied meat plant indicated that producers of meat products in Poland pay significant attention to quality and food safety of their products and also to the compliance with the law in force. The assessment of effectiveness of traceability system indicates that producers of meat products are ready in case of potential appearance of consumer health hazards. They would effectively remove the dangerous product from the market in the situation of hazards as well as the appearance of the product not compliant with the quality requirements. Simulations of hazards appearance on the market were carried out in the discussed plants and indicated that traceability systems in these plants are present and controlled all the time.

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4

THE RESULTS OF QMS AND HACCP IMPLEMENTATION IN THE OPINION OF THE EMPLOYEES OF STRAUSS CAFÉ POLAND COFFEE ROASTING PLANT

Introduction

Legal requirements in scope of food safety oblige the company to implement HACCP system. This system can be implemented, among others, according to following standards: Codex Alimentarius, ISO 22000:2005, IFS, BRC. Implementation of quality management systems is an effective and also more and more popular method used to achieve success on the market. Implementation of quality management systems is not obligatory, therefore an important motive for implementation is the will to increase export and market share as well as sustain the competitive position. Implementation of QMS can increase productivity of work through, among others, setting targets and scopes of responsibility to individual staff members. Thanks to increase of productivity as well as facilitate identification of nonconformities in products and services, the costs can be reduced.

In relation with that, the process of improvement, which results in waste and redundant work elimination as well as decrease in the number of complaints, can be started [Penc, 1999]. Several years of functioning of above mentioned systems in analyzed company gives a basis for an attempt to asses the results of implementation.

Target and methodology of the research

The target of conducted research was to canvass opinions of blue and white collar staff on the subject of QMS and HACCP systems implementation results in the coffee roasting plant STRAUSS CAFE POLAND Ltd in Swadzim, near Poznan. The surveyed organization has implemented an integrated quality management system based on the requirements of ISO 9001:2000 standard and HACCP system. During the research the company has been adapting to the ISO 22000:2005 standard. The survey was carried out in April 2008 amongst management staff and production workers. A sample of respondents was chosen randomly on the basis of quota sampling. Samples from management staff was collected separately from production workers samples.

Characteristics of researched population

The analysis of respondent's particulars shows the superiority in number of men in researched population in relation to number of women (M 69%, W 31%). On the other hand if we take age as the criterion, the largest group in the population were people between 41 and 45 years of age, and the smallest group was people over 51 years old – 16%.

In case of education, the number of respondents with secondary education prevailed – 53%. People with post-secondary school education were in vast minority – 3% (Figure 1)

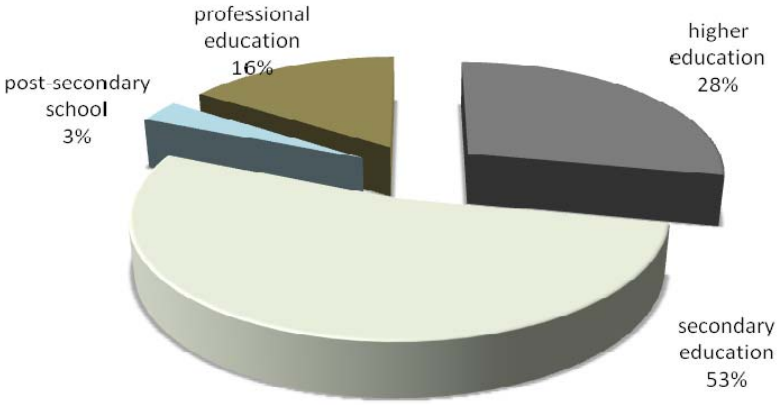

Fig. 1. Level of education in analyzed population

Source: Own description

A survey was conducted amongst respondents (managers – 12 people, production staff – 20 people), who constitute 31,07% of overall staff working in the company. A group of managers was constituted by, amongst others, production, marketing, accountancy, logistics and shift managers. 75% of the managers had a higher education, whereas 17% secondary education. In vast minority were people with post-secondary education – 8%. Further analysis of management staff shows, that the largest represented group were people in age between 20 and 30 as well as between 41 and 50 years of age – both groups 33%. Staff in age between 31 and 40 were in minority – 8%.

Blue collar staff included workers from storage and production areas. This group of respondents, similarly to mangers, mostly consisted of men (85%). Amongst the questioned staff 75% gained secondary education, remaining 25% professional education. Further analysis shows, that the largest represented group were people in age between 41 and 50 – 33%. Staff in age over 51 were in minority – 10% of questioned.

The positive results of QMS and HACCP system implementation in the opinion of the management and production employees

Each of the respondents could chose from sixteen positive and nine negative results of systems implementation. Because of the fact, that the analyzed questions were multiple choice questions, the amount of given answers is higher than the overall number of sample.

The respondents (both managers and production workers) have mostly indicated, that the improvement of an image of the company in the branch is a positive result of QMS implementation (QMS managers – 67%, QMS workers – 90%). This can have a reflection in the behaviour of clients, who express growth in the satisfaction from products. Moreover they feel that the company adjust greatly to their requirements, than before the implementation (Figure 2). As a consequence, the company could have gained new clients, by offering them highest quality products and services. Therefore on second place in respect of the number of indications made by questioned staff, the increase of products and services quality were pointed out (QMS managers – 58%, QMS workers – 95%). Third place in relation to the number of indications in both groups, the improvement in quality of work was stated (QMS managers – 58%, QMS workers – 45%). Pointing out this answer means, that the person working on certain position has a feeling of value of what he or she produces, and moreover the process is for him pleasant and valuable.

The analysis of positive effects of systems implementation shows, that the respondents often indicated, that the improvement of company functioning, mostly in scope of management concerning in highest rate proficiency and clarity of management. The staff is aware of their responsibilities, has clear tasks to conduct and sticks to them. In opinion of respondents (40% of managers) orderly documentation is also a great advantage of QMS. All procedures are kept in area of the department, which is perfectly known to the staff. Moreover the company has an internal computer system, in which the documents and recent records are stored. Depending on the authorities of every individual employee, the documents can be read, and finished as well as current tasks can be reviewed. The conducted research stated the increase in self-control of staff (50% of managers). Self--control is an element of quality control as well as an important motivation factor. Staff have more control over their actions and conducted work. The analysis shows that by implementation of QMS the shortages, complaints and nonconformities were reduced. Moreover thanks to reduction of number of mistakes the costs diminished. The presented positive results of QMS implementation show, that the management staff give more attention to office work and management aspects, such as improvement in control over documentation, increase in management efficiency over subordinate personnel, increase in self-control as well as proficiency and clarity of management. Production staff have minor contact with this system, therefore their activity during questions about the advantages of QMS was low.

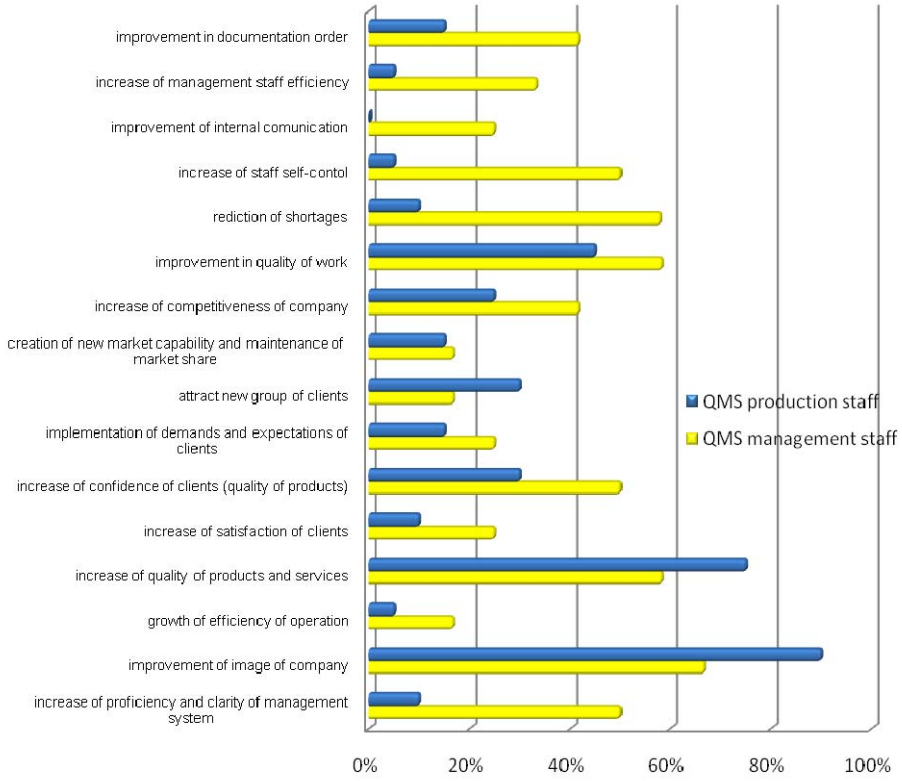

Fig. 2. Positive effects of Quality Management System implementation in management staff and production staff opinion (Source: Own description)

Amongst mostly chosen advantages of HACCP system, similarly to advantages of QMS, was improvement of the image of the company (HACCP managers – 92%, HACCP workers – 75%). Improvement of the image means that the relationships between the company and different participators of external environment improve. Image has a great value in making business, irrespective of its character. Good reputation of the company is a way to increase the advantage over the competitors and build long-term relations with other companies. According to the research, a great merit of HACCP system is increase in the products and service quality (HACCP managers – 75%, HACCP workers – 85%). It means, that the goods offered by the company are safe for the consumer and are of highest quality. According to respondents improvement in quality of work, especially in the opinion of production workers (60%) is a great positive effect of HACCP system implementation (Figure 3). It must be emphasized, that production workers indicated HACCP system more often than QMS. Probably it is due to everyday contact with this system by monitoring of CCP, complying with Good Manufacturing Practice (GMP) and Good Hygienic Practice (GHP). Another advantage of this system is an increase of self-control, which results from conduct consistent with the procedures and instructions, placed by their work stations.

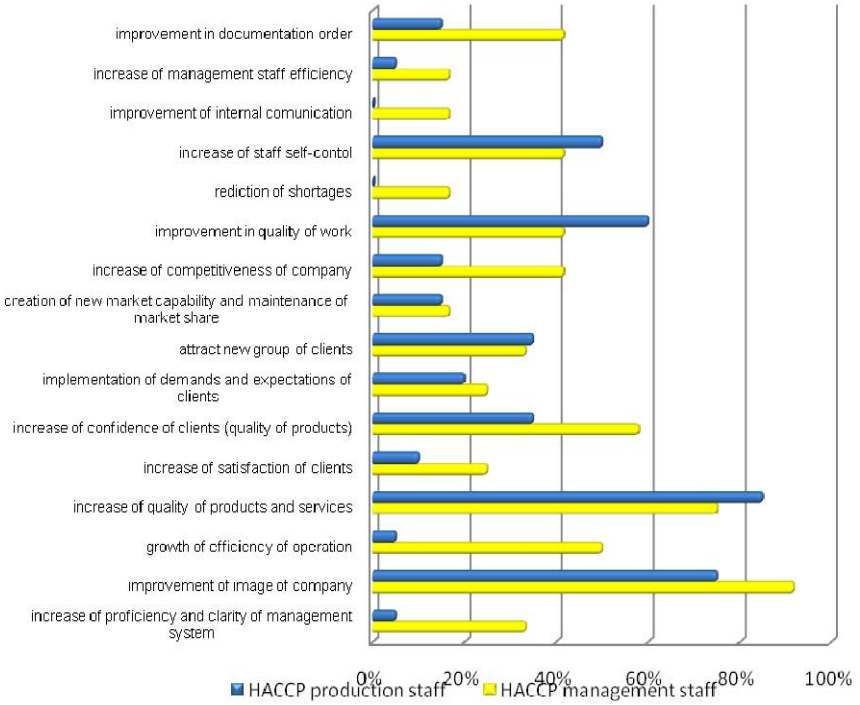

Fig. 3. Positive effects after implementing HACCP system in management staff and production staff opinion (Source: Own description)

The negative results of QMS and HACCP system implementation in the opinion of the management and production employees

Apart from advantages of implemented systems, which function as an Integrated Management System, the surveyed staff notice some disadvantages. The highest percentage of management staff respondents pointed out the increase in bureaucracy (QMS 58%, HACCP 50%), and the lowest indicated decrease in work productivity (QMS 8%, HACCP 0%) and efficiency (QMS 0%, HACCP 8%) (Figure 4). The negative results of systems implementation pointed out by the respondents are due to the requirements of ISO 9001 standard, which imposes the obligation to document certain procedures and maintain records, which are a confirmation of conducted activities. The increase of bureaucracy is caused by the fact, that earlier the companies did not have to document certain actions.

Similar conclusion can be made on the basis of indications concerning negative results of systems implementation given by production workers. They significantly emphasized the increase in bureaucracy (QMS 60%, HACCP 40%) (Figure 5). It can be claimed, that the negative impression of production workers and managers are alike.

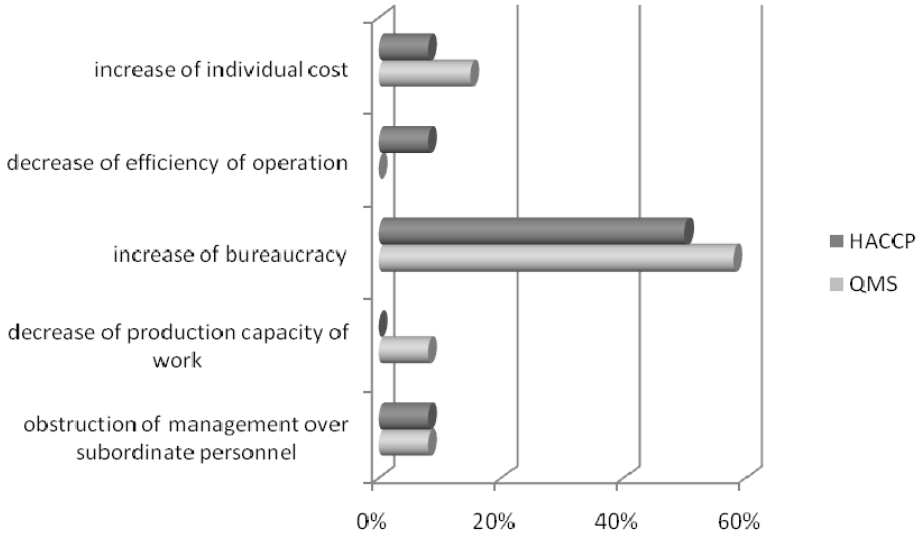

Fig. 4. Negative results of Quality management system and HACCP system in management staff opinion (Source: Own description)

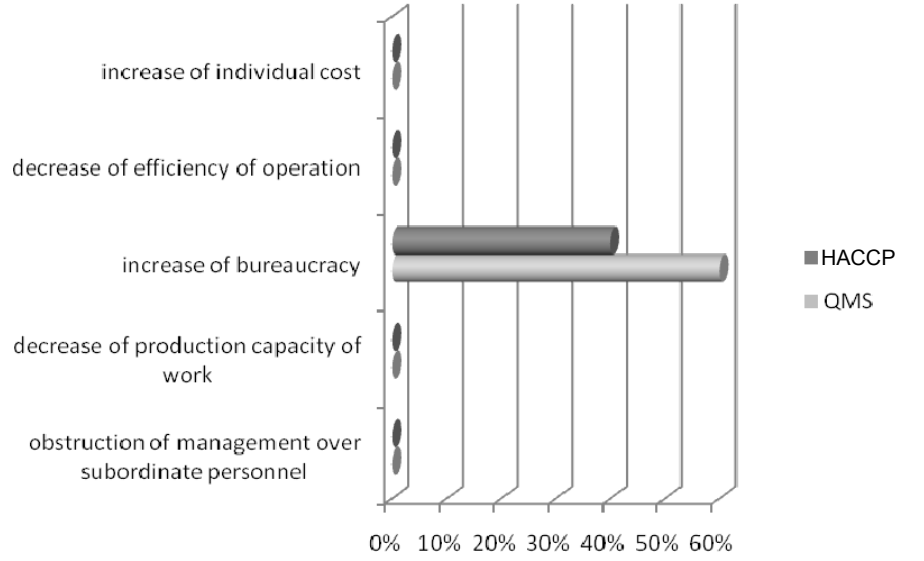

Fig. 5. Negative results of Quality management system and system HACCP in production staff opinion (Source: Own description)

On the basis of research results it can be perceived, that management staff and production workers have similar point of view on the systems, which function in the company. However each of this group attach greater value to the system, which they have contact with during their everyday activities. For management staff the quality management system is more important, which they work in the offices with, complying to the rules and conducting the processes in proper way. Whereas HACCP is a system within the framework of which the production staff conduct their everyday tasks at their workplace.

Conclusion

Present companies operate in more and more complex and dynamic environment. The pace of changes in production technology is speeding up, new markets are being opened and the requirements (especially concerning quality) are getting higher. The managers of many polish companies are put in new situation, in which they must verify the management methods they have used. Quality of products, which is needed to compete effectively on the market, should be the priority in conducted actions. Therefore in order to achieve demanded level of quality and food safety, QMS and HACCP systems are indispensable [Górna, 2008]. Conducted research in certain areas confirms the results of research on the efficiency of management system implementation carried out by Urbaniak. On the basis of conducted research Urbaniak determined, that implementation of HACCP system caused the greatest results in the field of food and processes safety, introduction of clear operational standards, decrease in number of the internal nonconformities and strengthening the positive image of the company in the market environment. It must be emphasized, that up to 92% of respondents amongst management staff and 75% of production workers of coffee roasting plant indicated (similarly to advantages of QMS), that HACCP system contributes to improvement of company image. On the other hand excessive bureaucracy of documents and records has been pointed out (also in Urbaniak's research) as the main barrier for quality management system and food safety management system implementation. Earlier the companies did not have to document certain actions, that is why after QMS and FSMS implementation they notice sudden increase of bureaucracy. It is necessary to ask a question: if thanks to these records the company is able to fulfill the requirements of the client, achieve repetitive quality of the product and in case of nonconformity is able to identify it and recall the product from the market quickly and ably, is not this apparent bureaucracy an advantage?

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CHAPTER 2

QUALITY OF FOOD RAW MATERIALS

1

QUALITY AND SAFETY ASPECTS OF SOME NEW GENERATION FOOD PRODUCTS IN LITHUANIA

Introduction

Many consumers are passionate about food, how and where it is produced, its quality, price and effect on their health. Consumers want food that contribute to a healthier lifestyle and that taste good, are convenient to prepare, and of course, are affordable. At the same time, European governments are struggling with the growing social and economic consequences of the alarming increase in obesity and related diseases. Consequently, food and dietary issues are now important topics of the debate throughout the European Union [Vision and strategic research agenda].

Proteins are particularly important material of human cells and play an important role in human nutrition. Amino acids content, their proportion and digestibility by humans define proteins biological value [FAO, 1991]. Proteins consist of 20 amino acids but the most important are essential amino acids what human body has to get with the food.

According to Aboul-Nasr [1997] and Tarek et al., [2001] amount of amino acids in plants depends not only on plant species, generative and vegetative part, but also on soil, climatic conditions, vegetation period. Sprouted wheat (*Triticum aestivum* L.) seeds, oil pumpkin (*Cucurbita pepo* L.) seeds and Jerusalem artichoke (*Helianthus tuberosus* L.) tubers are alternative, not widely spread, agricultural products containing important for human nutrition components. These products are valued not only for its nutritious and medical qualities but also for simple growing or sprouting technology. Protein content in Jerusalem artichoke varies from 2 to 3% [Tchoné, 2003]. Amino acids composition of Jerusalem artichoke tubers is little known. Cieślik [1998] reports that Jerusalem artichoke contains all essential amino acids in very advantageous proportions. Sprouted grains contain high amount of essential amino acids (lysine, methionine etc.), which take part in protein synthesis in human body [Tkachuk, 1979; Jahn-Deesbash, Schipper, 1991; Schöne et al., 1997].

Pathogenic and harmful microorganisms are widely spread in human environment, on food also. When growing and developing in food products microorganisms deteriorate their sensory quality, reduce nutrition value and pollute them with toxic chemical compounds. There have been applied harmless for humans preservatives to avoid infection of sprouted wheat grain with fungi mycelium and to prolong expiry period to use the sprouted grain as foodstuff [HN:53:2003]. Sprouted grains contamination with microorganism is one of the most important problems for food technologists. A successful grain decontamination treatment must inactivate microbial pathogens, while preserving seed viability, sprouting and vigour. For many years chemical disinfection agents are using for sprouted seeds decontamination with microorganisms. In the recent years scientists are looking for the novel

environmental and human friendly disinfection methods. Sorbic acid and calcium propionate, which are already used in food industry for avoiding pollution with microfungi and prolonging shelf life of numerous products, can be promising for sprouted seeds treatment as well.

The objective of this study was to find: local alternative plant raw materials rich in amino acids, and to elaborate novel, environment friendly, methods of reduction of fungi growth spread on wheat grains used for sprouting.

Material and methods

As the plant material we had chosen three species, which are promising as an alternative raw material for humans: oil pumpkin (*Cucurbita pepo* L.) – cultivars 'Danaja', 'Herakles' and 'Golosemiannaja', Jerusalem artichoke (*Helianthus tuberosus* L.) – 'Albik', 'Rubik' and line No. 05-7, wheat (*Triticum aestivum* L) - 'Zentos' and 'Sirvinta 1'. Oil pumpkin seeds and seed cake, Jerusalem tubers and wheat sprouted grains quality were taken to the research. Plant material was obtained in the Experimental Station of the Lithuanian University of Agriculture. The plants were cultivated on a limnoglacial loam of the following characteristics: pH slightly acid or neutral, medium humus content, phosphorus–rich and potassium–rich. Pumpkins were harvested in the last decade of September, tubers of Jerusalem artichoke in the first decade of November and winter wheat grain – in the end of July.

All plant samples (pumpkins seeds and cakes, tubers of Jerusalem artichoke, winter wheat sprouted grain) were subjected to the analysis of amino acids.

Analytical methods. Amino acids were separated by the method of ion-exchange chromatography and quantified photometrically by measuring of absorbance at 570 nm with automatic amino acids analyzer Mikrotechna AAA 339, using glass column (∅ 0.37 × 45 cm), filled with ionite Ostion LGANB. Hydrolysis of the sample was performed in the presence of 6 M HCl at 105°C, for 24 h. The essential amino acids: threonine, valine, methionine, isoleucine, leucine, phenylalanine, histidine, lysine, arginine, tryptophan and non-essential amino acids: aspartic acid, serine, glutamic acid, glycine, alanine, tyrosine, proline, cysteine were determined [Technical regulation of amino acids ..., 2003].

Grain sprouting method. Grains were sprouted in „Bio–natura“ sprouters (plate capacity – 1 L, diameter – 20 cm) in the dark, for 120 hours. For sprouting 250 g of grains have been washed and imbibed for 12 hours in water (ratio 1:4).

Grain disinfection

I experiment. Grains disinfection was performed using 6% hydrogen peroxide and 1% grapefruits seeds extract. In this solutions grains were kept for 15 min. After disinfection grains were washed in sterile water and sprouted for 120 hours.

Determination of grain contamination with fungi. Quantitative grains contamination with fungi (cfu/g) has been determined by examining the washed seeds liquid [Smirnova, Kostrova, 1989].

II experiment. 5-Aminolevulinic acid (ALA), the precursor of endogenous photosensitizer protoporphyrin IX – was provided by Photo Cure (Oslo, Norway). Stock solutions (2.5×10^{-2} M) were prepared and sterilized the same day as they were used. Grains were placed in sterile tubes (45 ml) and incubated with ALA for 4 hours at the temperature of

26°C. For all experiments ALA concentration was $6x10^{-3}$ M. Afterwards the grains were placed in sterile Petri plates and irradiated. Following 12 hours microbiological analysis were performed of untreated (control), ALA incubated, ALA incubated and irradiated grains (PDT – photodynamic technology). The grains were irradiated with non-coherent light source, based on 400 W incandescent lamp, equipped with optical systems to deliver light with several wavelengths, at power density 40 mW/cm^2. Irradiation time was optimized earlier 15 min. Total energy which reached the surface of the grains during irradiation was 36 mJ/cm^2 [Lukšienė, 2005; Lukšienė et al., 2007].

Determination of grains contamination with fungi. Percentage of internal damage and external infection of raw grains by fungi was investigated by the method of Smirnova and Kostrova [1989]. When determining internal damage by fungi, the grains were inserted into bags and disinfected with 70° ethyl alcohol for 3 min. After disinfection, the bags containing grains were rinsed with sterile water. Internal damage and external infection by fungi was determined by sowing 100 grains of every sample into Petri plates with agarized synthetic Czapek medium. Fungi were grown in thermostat at temperature of 26±2°C. Fungi colonies were calculated on the 3rd, 5th, and 7th day of incubation. Genera of fungi were identified according to descriptions by Domsch et al. [1980], Nelson et al. [1994], Samson and van Reenen-Hoekstra [1988] and Lugauskas et al. [2002].

III experiment. Preserves calcium propionate (Rietmann GmbH, Germany) and sorbic acid (Jiali International Corporational Corporation, China) were used in the concentration of 1.5 and 2% on sprouted wheat grains weight, accordingly to the references of Lithuanian hygiene norm [HN:53:2003].

Determination of grains contamination with fungi. Fungi mycelium growth was determined and fungi species identification in sprouted and non- sprouted wheat grains was performed. Method of fungi species separation was based on product and its solution sowing into agar breeding – ground of beer mash. Growth of fungi colonies was determined on third, fifth and seventh days of their development, accordingly to references of LST ISO 21527:2008. There were separated monocultures from fungi colonies by microscopy method. These monocultures were identified according to Domsch et al. [1980], Samson [1988].

The data were evaluated by ANOVA, using program STATISTICA. All determinations were done in triplicates. Means and standard errors of the experimental data were calculated. Statistical reliability among data was evaluated according to Fisher LSD test. Difference were statistically proved when $p<0.05$ [Sakalauskas, 2003].

Results and discussion

Pumpkins. According to Tarek [2001] and Jariene et al. [2007] amount of amino acids in plants depends on plant species, part of the plant (generative or vegetative), soil, climatic conditions and vegetation period. Oil pumpkin seeds and cakes contained about 32% of crude protein [Peričin et al. (a), 2009; Peričin et al. (b), 2009]. The analysed seeds and oil cake contained 16 identified amino acids, of which 9 were essential ones and 7 – non-essential ones (Table 1). Comparing the amount of amino acids in various pumpkins cultivars the highest amount of essential and non-essential amino acids accumulated in 'Danaja' seeds and 'Golosemiannaja' seeds cakes. In all pumpkin cvs. seeds and cakes the most dominated essential amino acid was arginine (Table 1 and Table 2). The biggest amount of

arginine was found in 'Golosemiannaja' seeds, and the lowest amount in 'Herakles' seeds, respectively 52.7 and 79.0 and 51.2 and 65.0 g kg $^{-1}$ dm. The non-essential glutamic acid dominated in all cv. pumpkins seeds and cakes. 'Herakles' seeds and 'Golosemiannaja' seeds cake are the richest in glutamic acid – 63.1 and 92.9 g kg $^{-1}$ dm, respectively (Table 1 and Table 2).

Table 1

Amount of essential and nonessential amino acids in pumpkin seeds [g kg $^{-1}$ dm]

Amino acid	'Danaja'	'Herakles'	'Golosemiannaja'
Essential amino acids			
Arginine	51.99 ± 0.09	51.19 ± 1.15	52.71 ± 0.25
Lysine	18.90 ± 0.02	18.39 ± 0.04	18.26 ± 0.04
Histidine	11.60 ± 0.04	10.84 ± 0.08	11.13 ± 0.04
Phenylalanine	16.19 ± 0.04	15.95 ± 0.06	15.40 ± 0.04
Leucine	21.48 ± 0.23	21.15 ± 0.30	20.17 ± 0.04
Isoleucine	12.40 ± 0.05	11.96 ± 0.04	11.85 ± 0.16
Methionine	9.37 ± 0.04	9.53 ± 0.04	9.57 ± 0.05
Valine	14.41 ± 0.01	14.01 ± 0.05	13.85 ± 0.05
Threonine	9.90 ± 0.04	10.00 ± 0.10	9.63 ± 0.03
Nonessential amino acids			
Tyrosine	10.35 ± 0.02	10.27 ± 0.02	9.97 ± 0.02
Alanine	14.99 ± 0.07	14.42 ± 0.05	14.49 ± 0.15
Glycine	19.55 ± 0.01	18.04 ± 0.08	17.73 ± 0.19
Glutamic acid	62.45 ± 0.18	63.16 ± 1.21	62.16 ± 0.36
Serine	16.76 ± 0.19	16.19 ± 0.02	15.82 ± 0.04
Aspartic acid	27.88± 0.18	27.25 ± 0.05	26.89 ± 0.04
Proline	11.82 ± 0.08	11.90 ± 0.03	11.97 ± 0.09

Table 2

Amount of essential and nonessential amino acids in pumpkin seeds cake [g kg $^{-1}$ dm]

Amino acid	'Danaja'	'Herakles'	'Golosemiannaja'
Essential amino acids			
Arginine	67.38 ± 1.09	65.01 ± 0.06	79.09 ± 1.29
Lysine	23.12 ± 0.02	22.31 ± 0.05	26.25 ± 0.01
Histidine	15.05 ± 0.04	14.16 ± 0.03	17.30 ± 0.03
Phenylalanine	20.17 ± 0.25	19.67 ± 0.05	22.62 ± 0.08
Leucine	28.49 ± 0.74	26.90 ± 0.07	31.64 ± 0.53
Isoleucine	16.69 ± 0.16	15.55 ± 0.16	18.31 ± 0.15
Methionine	12.67 ± 0.09	12.41 ± 0.04	14.48 ±0.15
Valine	18.35 ± 0.20	17.84 ± 0.11	21.02 ± 0.09
Threonine	13.07 ± 0.04	12.59 ± 0.02	14.65 ± 0.09
Nonessential amino acids			
Tyrosine	12.48 ± 0.04	12.49 ± 0,13	14.79 ± 0.08
Alanine	19.35 ± 0.19	18.45 ± 0,06	22.10 ± 1.04
Glycine	24.24 ± 0.01	23.20 ± 0,31	26.65 ± 0.09
Glutamic acid	80.93 ± 1.32	78.46 ± 2,14	92.91 ± 1.37
Serine	21.63 ± 0.09	21.33 ± 0,49	23.89 ± 0.15
Aspartic acid	35.52 ± 0.36	35.01 ± 0,17	40.95 ± 0.08
Proline	15.16 ± 0.04	14.47 ± 0,05	16.89 ± 0.08

Jerusalem artichoke. The content of amino acid in Jerusalem artichoke tubers mostly depends on a cultivar, as well as on growing and storage conditions [Stolzenburg, 2004]. Arginine was the dominating essential amino acid in tubers of Jerusalem artichoke. Amount of arginine was almost 4-times higher than of the other ones (Table 3). Comparing amounts of essential amino acids in different Jerusalem artichoke cultivars tubers, 'Albik' tubers were higher in amino acids content, although in 'Rubik' and No. 05-7 tubers the amount of amino acids was only 1.8–1.9 g kg $^{-1}$ less than in 'Albik' (Table 3). The biggest amount of all non-essential amino acids was determined in 'Albik' tubers (Table 3). The prevailing non-essential amino acid in Jerusalem artichoke tubers was glutamic acid, and its amount, comparing with other nonessential amino acids, was 4–5 times higher. The lowest amount was determined in the case of tyrosine. In 'Albik' tubers, amount of glutamic acid was 11.1 g kg^{-1}, while in 'Rubik'– 7.0 g kg^{-1} dm (Table 3). The essential and total amino acids ratio in Jerusalem artichoke tubers was higher than that reported (33.9%) [FAO, 1991]. This ratio in tubers of 'Albik' was 54.1%, 'Rubik' – 56.8% and line No. 05-7 – 54.9%.

Table 3

Amount of essential and non-essential amino acids in Jerusalem artichoke tubers [g kg $^{-1}$ dm]

Amino acids	Cultivar / line		
	'Albik'	'Rubik'	No. 05-7
Essential amino acids			
Arginine	9.57±0.34	8.64±0.02	8.41±0.03
Lysine	2.97±0.02	2.74±0.02	2.56±0.02
Histidine	1.42±0.02	1.31±0.02	1.45±0.01
Phenylalanine	1.98±0.01	1.91±0.04	1.99±0.01
Leucine	2.92±0.02	2.82±0.02	2.86±0.04
Isoleucine	3.10±0.03	2.89±0.01	2.84±0.02
Methionine	0.90±0.01	0.89±0.02	0.77±0.02
Valine	2.36±0.01	2.27±0.02	2.84±0.03
Threonine	2.09±0.02	2.02±0.02	2.16±0.02
Nonessential amino acids			
Tyrosine	0.95±0.02	0.96±0.05	0.96±0.02
Alanine	2.81±0.03	2.73±0.03	2.77±0.01
Glycine	2.33±0.01	2.31±0.04	2.46±0.02
Glutamic acid	11.09±0.03	7.03±0.05	8.31±0.03
Serine	1.98±0.01	2.13±0.04	2.10±0.02
Aspartic acid	3.96±0.03	4.18±0.03	4.23±0.03

Sprouted wheat grains. There has been determined that sprouting influenced the most positively upon synthesis of arginine and phenylalanine, also upon glicine, alanine, glutamic and aspartic amino acids (Table 4). That increase can be explained by photosynthesis process and more intensive hydrolysis of proteins at darkness, also by increased activity of proteases. Two-day sprouting process had the strongest influence upon arginine (in 'Zentos' and 'Širvinta 1' – 10.5% and 10.8%, respectively) and phenylalanine (in 'Zentos' and 'Širvinta 1' – 10.6% and 10.5%, respectively) and glycine, hystidine have been affected at least (2.8%) and glutamine has been influenced meanly (3.6%) (Table 4). Our research show statistically reliable influence of wheat cultivar upon serine, glutamine and proline amino acid content in grains.

Table 4

Amounts of essential and nonessential amino acids in non-sprouted and sprouted wheat grains [g·kg^{-1} dm]

Amino acids	Cultivar			
	'Zentos'		'Širvinta 1'	
	Non-sprouted	Sprouted	Non-sprouted	Sprouted
Essential amino acids				
Arginine	5.06±0.08	5.36±0.06	4.93±0.01	5.36±0.02
Lysine	2.84±0.04	3.03±0.01	2.92±0.01	3.11±0.03
Histidine	2.61±0.02	2.66±0.01	2.58±0.02	2.66±0.03
Phenilalanine	4.92±0.03	5.21±0.03	4.73±0.03	4.97±0.04
Leucine	7.41±0.04	7.56±0.02	7.64±0.03	7.88±0.02
Isoleucine	4.31±0.02	4.32±0.03	4.21±0.01	4.39±0.04
Methionine	1.75±0.03	1.78±0.02	1.75±0.02	1.86±0.03
Valine	5.12±0.05	5.25±0.01	5.18±0.01	5.36±0.01
Threonine	3.16±0.11	3.27±0.03	3.08±0.01	3.26±0.01
Tryptophan	1.19±0.01	1.25±0.03	1.29±0.03	1.33±0.02
Nonessential amino acids				
Tyrosine	3.05±0.01	3.03±0.03	2.93±0.03	3.03±0.04
Alanine	3.95±0.01	4.25±0.01	4.03±0.01	4.25±0.02
Glycine	4.28±0.04	4.53±0.01	4.22±0.02	4.53±0.01
Glutamic acid	30.84±0.01	31.17±0.01	29.78±0.01	30.83±0.01
Serine	5.05±0.02	4.98±0.02	4.77±0.02	4.98±0.04
Aspartic acid	5.44±0.01	5.75±0.03	5.64±0.04	6.26±0.03
Proline	10.59±0.02	10.76±0.04	10.42±0.02	10.56±0.02
Cysteine	2.69±0.03	2.95±0.03	2.71±0.03	2.95±0.01

Reducing of fungi contamination of grains used for sprouting as a food, using novel environment friendly technology

I experiment. Mycological raw seeds contamination has significantly decreased after disinfectants application. However, significant difference between mycological contamination in grains disinfected using grapefruit seeds extract and hydrogen peroxide has not been found (Fig. 1). After 120 h of sprouting of not disinfected grains, the contamination decreased comparing with not disinfected. Fungi growth reduction may be related to the grains rinsing during sprouting time and to the specific anti-fungi compounds existing in the grains. Significant changes in fungi contamination were found in sprouted undisinfected and disinfected seeds. After disinfection with H_2O_2, fungi in sprouted grains were not detected (Fig. 1).

II experiment. Taking into account that photosensitization, based bio photonic technology, might decontaminate seed of different shape, size and geometry, it was designed other experimental setup, based on endogenous production of photo-sensitizer in mycellium without any harm effect on food matrice. It is well-known that 5-aminolevulinic acid (ALA) is a precursor of endogenous photo-sensitizer protoporphyrin IX (PpIX). Incubating grains with ALA, which is un-mutagenic, un-cancerogenic or genotoxic, at 26°C, we allowed mycellium to accumulate ALA and to produce endogenous PpIX inside the cells.

After irradiation with visible light we observed decreased fungi contamination of wheat grain. Mycelliums of *Fusarium* spp., *Aspergillus* spp., *Mucor* spp., *Rhizomucor* spp. were susceptable to this treatment and only *Acremonium and Cladosporium* spp. were resistant. (Fig. 2 and Fig. 3). According to Luksiene [Luksiene et al., 2007] ALA induces either marginal or significant activities of antioxidant enzymes which can be associated with enhanced cellular capacity to detoxify reactive oxygen species.

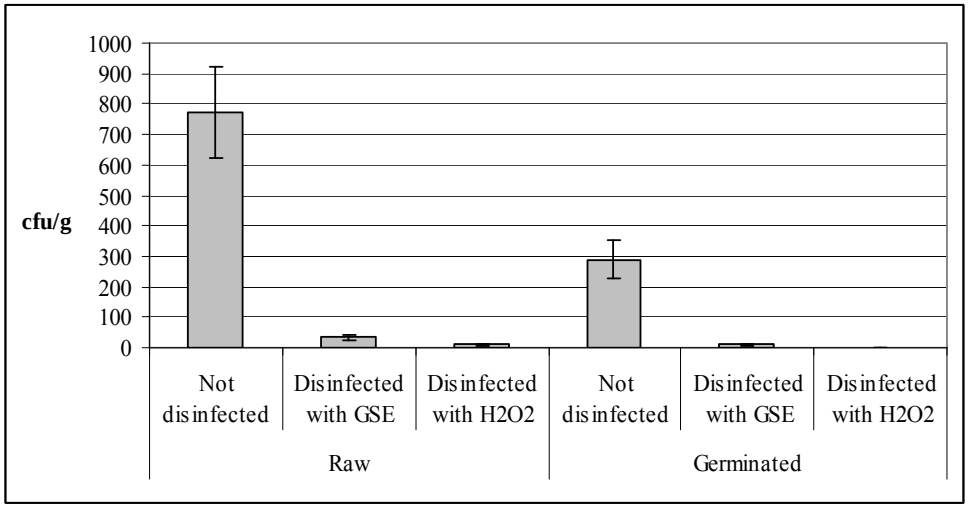

Fig. 1. Raw and sprouted wheat grains contamination with microfungi after grapefruit extract treatment (GSE) and hydrogen peroxide (H_2O_2) solutions (Values are means with standard deviation)

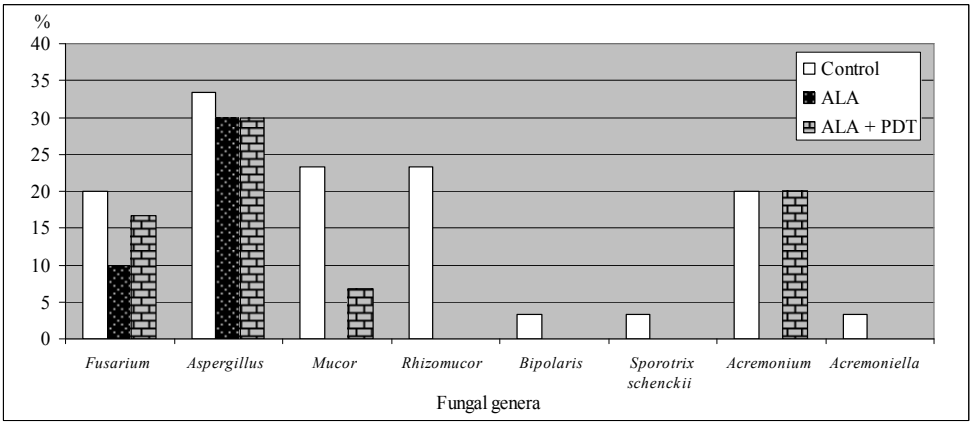

Fig. 2. External fungal infection of wheat grains before and after treatments

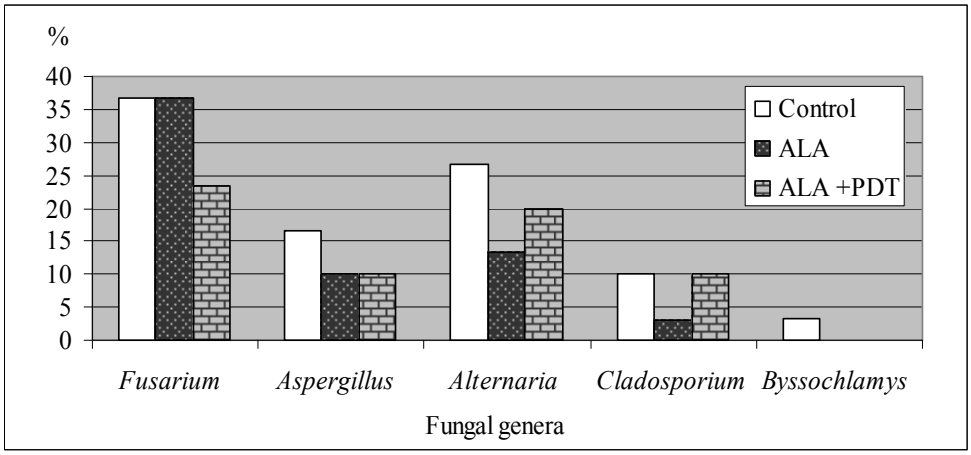

Fig. 3. Internal fungi damage of wheat grains before and after treatments

III experiment. Data in literature indicate that only high concentrations of propionic acid and its salts block activity of enzymes and suppress development fungi mycelium [Abu-Ghoush et al., 2008]. Besides, antimicrobial efficiency of propionic acid depends on pH of environment, while it was not found any influence of sorbic acid and its salts. It was confirmed in this study. Calcium propionate did not suppress the growth of fungi, while sorbic acid acted as suppressant in many cases (Table 5). Inhibition of fungi growth was determined in all analyzed samples, when maximum allowable concentration of sorbic acid was used (2.0 g kg^{-1}). That preservative is effective even at lower concentrations.

Table 5

The influence of preservative concentration and storage time on microfungi number variation on sprouted wheat grains

Sample name	Concentration of preservative	Number variation of microfungi cfu g^{-1}		
		Storage time of wheat grains		
		1 day	3 days	7 days
Sprouted wheat grain with sorbic acid	1.5 g kg^{-1}	0	5.5 x 10^1	3.5 x 10^2
	2 g kg^{-1}	0	2.5 x 10^1	1.9 x 10^2
Sprouted wheat grain with calcium propionate	1.5 g kg^{-1}	2.0 x 10^3	2.7 x 10^4	>150
	2 g kg^{-1}	1.8 x 10^3	3.6 x 10^4	>150
Sprouted wheat grain without preservative	–	2.1 x 10^3	7.6 x 10^4	>150

Conclusions

Amount of amino acids in all plants material depended on plant species. Pumpkins are markedly richer of amino acids than Jerusalem artichoke and sprouted grains. Essential amino acids arginine and leucine also non-essential glutamic acid dominated in pumpkin seeds and seeds cakes. Due to the higher content of amino acids investigated pumpkin cultivars should by recommended as important component of human diet.

Biophotonic ecologically friendly technology seems suitable for inactivation of some microfungi genera. Decontamination of wheat with hydrogen peroxide and grapefruit seeds extract was effective, even after seeds sprouting process. Therefore using that decontamination method the shelf life of sprouted grains can be markedly increased and the product will be safe as a food. Sorbic acid as a preservative is an effective inhibitor of microfungi multiplication even at lower concentration.

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2

THE INFLUENCE OF CULTIVARS AND FERTILIZERS ON VITAMIN E CONTENT IN OIL PUMPKIN (*Cucurbita pepo* L.) SEEDS AND OIL

Introduction

Oil derived from pumpkin seeds is a rich red-green colour, and is considered by many people to be quite tasty. Pumpkin seed oil is sold as a 'healthy food' because of its remarkable nutritional value, and is often added to beverages or food to enrich them and confer some additional health benefits. The primary benefits from pumpkin seed oil intake by human include improved brain function and energetic processes stimulation. It also supports the action of prostate gland [Sawaya et al., 1983; Murkovic et al., 1999].

In Lithuania, pumpkins are being discovered as a vegetable. So far they have been used for feed or grown as ornamental plants. Oil extracted from the seeds of oil pumpkins with a cold pressing is still a novelty, although imported pumpkin oil has been sold in supermarkets for several years. Oil pumpkins originate from south Austria (Stiria region). Areas of pumpkin cultivation have been constantly increasing in Czech Republic, Hungary and south Ukraine. This vegetable is ranked as valuable food in many countries. For instance, in Japanese ranking of foodstuff value, pumpkin seeds are situated on the first place. Pumpkin seeds are perfect material for oil production, to be used not only as a food, but also in medicine. Human populations in western Ukraine, Hungary, Austria, Romania and Italy use pumpkin oil as a food additive [Lazos, 1986; Elmadfa, Fritzsche, 1999; Ekpedeme et al., 2000].

Vitamin E, present in large quantities in pumpkin seed oil, is a very active antioxidant, particularly in the form of γ-tocopherol. Antioxidants are known to be effective in the prevention of several civilization diseases, such as cancer and heart diseases, mainly due to their ability to inhibit the action of oxidants (free radicals), which are potentially harmful for the body cells. Free radicals cause hazard by binding themselves to otherwise healthy tissue and destroying it. Sometimes this can cause cancer and the growth of a tumour. Antioxidants help to prevent this build-up of plaque, and therefore to ward off future cardiovascular problems.

The γ-tocopherol present in pumpkin seed oil, in addition to being an antioxidant, also possesses anti-inflammatory properties that can be used to treat arthritis and other conditions which cause painful swelling. Comparisons between pumpkin seed oil and latest drug treatments for arthritis indicate that in many cases pumpkin seed oil shows a greater remedy and anti-inflammatory action than the chemical drugs [Ekpedeme et al., 2000; Bavec et al., 2002; Nkosi et al., 2006].

Application of fertilizers influences the natural environment and marketable, nutritional and technological characteristics of plant products. A big assortment of fertilizers, available on the Lithuanian market, increases interest in the influence of organic and mineral fertilizers on chemical composition of pumpkins and their impact on fruit and seed quality. Different literature sources recommend fertilizing pumpkins with different complex fertilizers with macro and microelements. For the basic fertilization multipurpose garden fertilizers with N:P:K ratio of 10:10:20 are used. These are granular complex fertilizers containing optimum amounts of the main macro- and microelements (N:P:K = 10:10:20 + 4.2% MgO, 11% S, 0.15% B, 1% Ca, 0.1% Fe, 0.7% Mn, 0.01% Mo, 0.1% Zn, 0.001% Se). Pumpkin fertilization at a rate of 500–700 kg ha^{-1} is usually applied [Michael, 2001; Lundergardh, Martensson, 2003]. However, little is known how complex mineral fertilizers affect biochemical composition of pumpkin seeds.

Recently, increasing attention has been paid to organic fertilizers. Among them there are humus acids fertilizers. Humus acids fertilizers (N:P:K = 0:0:0) are products of humic acids, which are found in nature as humus and peat components [Stevenson, 1994]. The effects of humus and humic acids on the plant are various. Firstly, humic acids perform physiological function and are ranked as growth stimulators, stimulating root development, plant growth, amount of chlorophyll in the leaves, intensify respiration and photosynthesis. They improve absorption of nutrients from the soil, reduce intensity of chemical sorption, therefore phosphorus available for plants with higher amounts of humic acids less interlink into inaccessible combinations. From the ecological standpoint, humus acid fertilizers can be used to reduce the negative effects of pesticides on plants (humic acids interlink toxic elements, radioactive isotopes among them into little mobile combinations) and accelerate degradation of the pesticide residues into non-toxic compounds [Lundergardh, Martensson, 2003; Michael, 2001]. However, little is known about the effect of organic (humus acids) fertilizers on pumpkins and their seeds.

The aim of the work was to determine the influence of different fertilizers on vitamin E amount in seeds and in oil of different cultivars of oil pumpkin (*Cucurbita pepo* L.).

Material and methods

Pumpkin cultivars ‘Miranda’, Golosemianaja’ and ‘Herakles’ were grown in the experimental field of Lithuanian University of Agriculture (LUA) in 2005–2006. The plants were planted out in the second half of May and harvested at the end of September. Different fertilization methods were applied: 1 – control (without fertilizers); 2 – humic acid fertilizers (the dose was calculated according to necessary Humistar amount) – 30 l ha^{-1}; 3 – complex fertilizers (N:P:K – 10:10:20) – 500 kg ha^{-1}; 4 – compost (70% manure + 30% composted plant wastage) – 40 t ha^{-1}; 5 – fertilizers mixture (complex fertilizers (N:P:K – 10:10:20 + humic acid fertilizers – 500 kg ha^{-1} + 30 l ha^{-1}). Humus acid fertilizers were sprayed on the surface of the soil, whereas complex fertilizers and compost were spread and interposed into soil on each plot.

Analysis of various indices brings soil of the entire experimental area to the same group of fertility, therefore, in all replicate blocks the treatments were situated systematically.

Primary total size of experimental plot was 6.0 x 6.0 m, width of protective belt – 4.0 m, size of accounting plot – 4.0 m^2. Cluster sowing method was applied and three seeds were put into one hole of 8 cm depth.

Pumpkins were grown in the soil with the following characteristics: acidity slightly neutral and neutral, medium humus content, phosphorus – rich and potassium – rich. In the experimental area the soil was drained by drainage, the relief was artificially levelled.

In seeds of mature pumpkin fruits there were determined:

- crude fat content – by extraction method;
- tocopherols (α-, β-, δ- and γ-) have been separated by high performance liquid chromatography (HPLC) on Pinacle II silica 5 μm particle size, 4.6 x 150 mm column, according to the method described by Murkovic et al. [1999]. Analyses were performed with Shimadzu HPLC 10A system.

Analyses were carried out in TEMPUS and chemistry laboratories of Lithuanian University of Agriculture and at Laboratory of Plant Physiology of Lithuanian Institute of Horticulture. Soil analyses were carried out in the centre of Agrochemical investigations of Lithuanian Institute of Agriculture. The experimental data was statistically processed with the ANOVA, software STATISTIKA [Sakalauskas, 2003].

Results and discussion

Such factors as geographical location, species and processing technique may influence the final chemical composition of plant oils [Beardsell et al., 2002]. The oil content in pumpkin seeds is reported to be in amount of 40–50% [Murkovic et al., 1999]. Oil pumpkin seeds are perfect material for oil production. There are reports suggesting that up to 37.8–50.0% of crude fats can be found in the seeds of oil pumpkins [Tarek et al., 2001]. In our study, the amount of crude fats in seeds of different pumpkin cultivars ranged from 42.8 to 45.7% (Fig. 1). Seeds of ‘Miranda’ contained the lowest amount of crude fats (42.8%). The highest fluctuation of these compounds was observed in ‘Herakles’ seeds (Fig. 1).

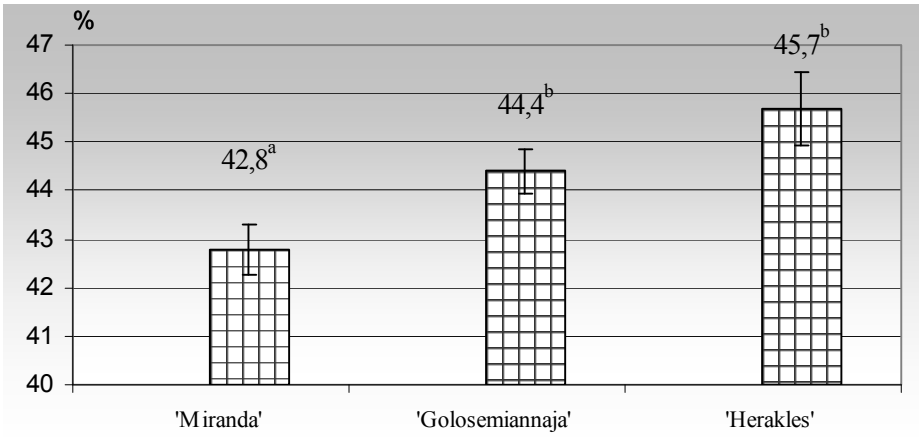

Fig. 1. Amount of crude fat in oil pumpkin seeds [%], LUA (means of 2004–2006)

Phosphorus, nitrogen and potassium fertilizers are particularly important for fats synthesis in seeds. For this reason fats content may increase by 2–4% and even more. It is assumed that fats content in pumpkin oil seeds also depends on the composition of fertilizers, i.e. phosphorus fertilizers increase the fats content, while the nitrogen fertilizers – reduce it [Tarek at al., 2001; Oshima et al., 1996]. The above statement was not confirmed in this work. We found that the used fertilizers had no significant effect on the crude fats synthesis in the seeds of 'Miranda' and 'Golosemiannaja' cultivars. Significantly higher amounts of crude fats were accumulated in seeds of pumpkins fertilized with compost – 45.7% (Fig. 2). Humic acid fertilizers significantly reduced the amount of crude fats in seeds – to 42.4% (Fig. 2).

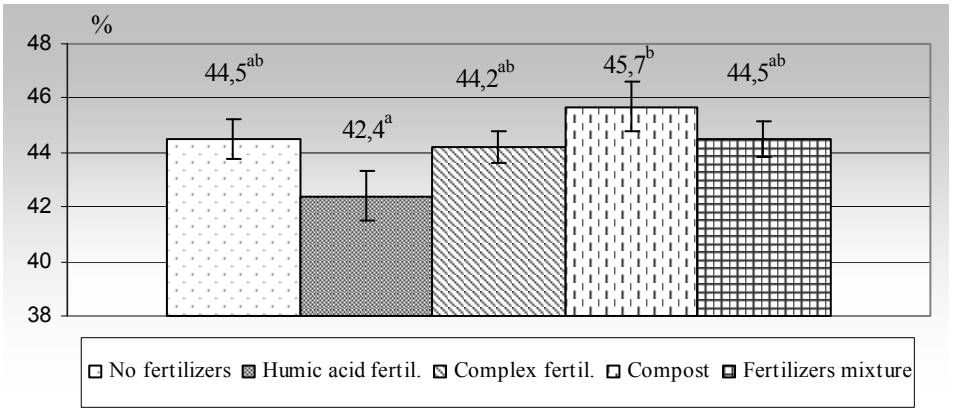

Fig. 2. Influence of fertilizers upon the amount of crude fats in oil pumpkin seeds [%], LUA (means of 2004–2006)

The amounts of different vitamin E isomers varied in different plants and their seeds. In comparison with other regularly used oils, the cold-pressed pumpkin oil is unique for the big amount of α-, β- and γ-tocopherol isomers. According to Murkovic et al., (1999], γ-tocopherol dominated in oil pumpkin seeds and its amount ranged from 41 to 620 mg kg^{-1}. Amounts of α-, β- and γ-tocopherol isomers were slightly lower – from 0 to 140 mg kg^{-1} and from 16 to 49 mg kg^{-1}, respectively. In other studies, some changes in biochemical and agronomical characteristics were determined in medicinal pumpkin (*Cucurbita pepo* L.) plants treated with plant growth regulators. Sedghi et al. [2008] reported that the accumulation of γ–tocopherol in the seeds of GA3 treated plants was about 19.5% higher than in the control, and reached the value of 220.2 mg kg^{-1} dm.

Data obtained showed that the lowest amount of δ-tocopherol isomers in pumpkin seeds was 90–122 times less in comparison with the highest amount of γ–tocopherol isomers (Fig. 3). In different cultivars, the largest amount of γ-tocopherol isomers ranged from 279.9 to 356.5 μg g^{-1}, α-tocopherol – accordingly from 108.3 to 150.5 μg g^{-1}, and β-tocopherol – from 144.7 to 214.2 μg g^{-1} (Fig. 3). The amount of γ-tocopherol in seeds depended upon cultivar and the largest amount were found in cv. 'Miranda' and 'Golosemiannaja' seeds (3546 and 3565 μg g^{-1}, respectively) and the smallest – in 'Herakles' (279.9 μg g^{-1}) (Fig. 3).

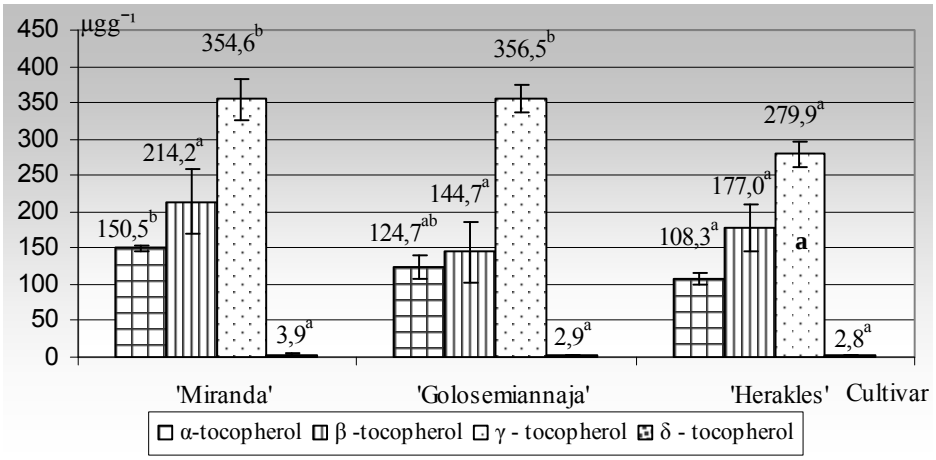

Fig. 3. Amount of tocopherol isomers in oil pumpkin seeds [µg g^{-1}], LUA (means of 2005–2006)

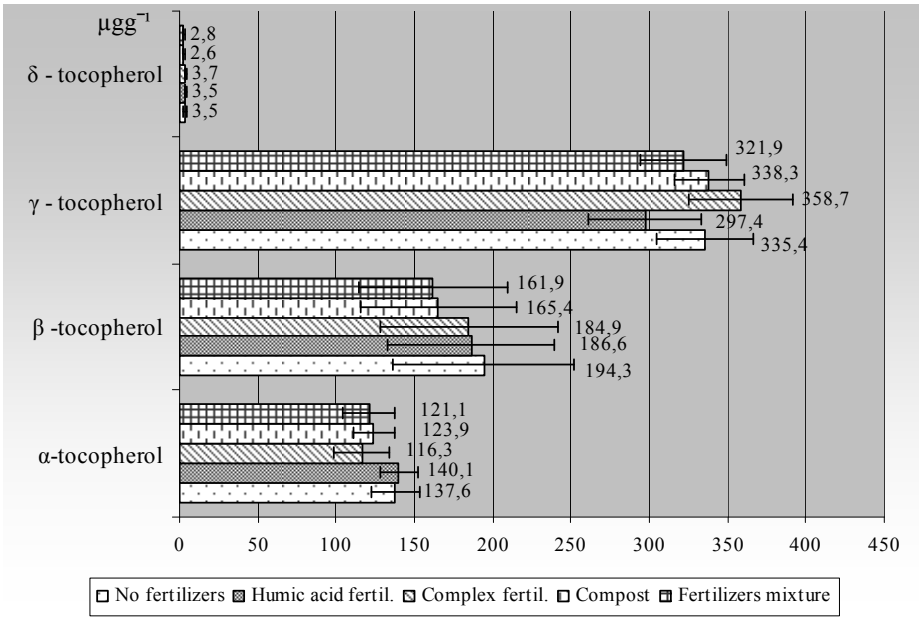

Fig. 4. Influence of fertilizers on tocopherol isomers content in oil pumpkin seed [µg g^{-1}], LUA (means of 2005–2006)

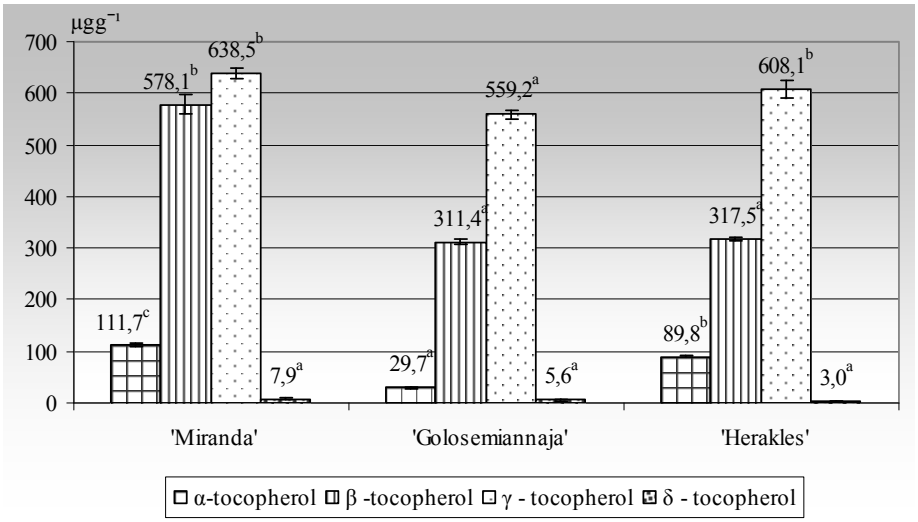

Fig. 5. Amount of tocopherol isomers in oil pumpkin seeds oil [µg g^{-1}], LUA (means of 2005–2006)

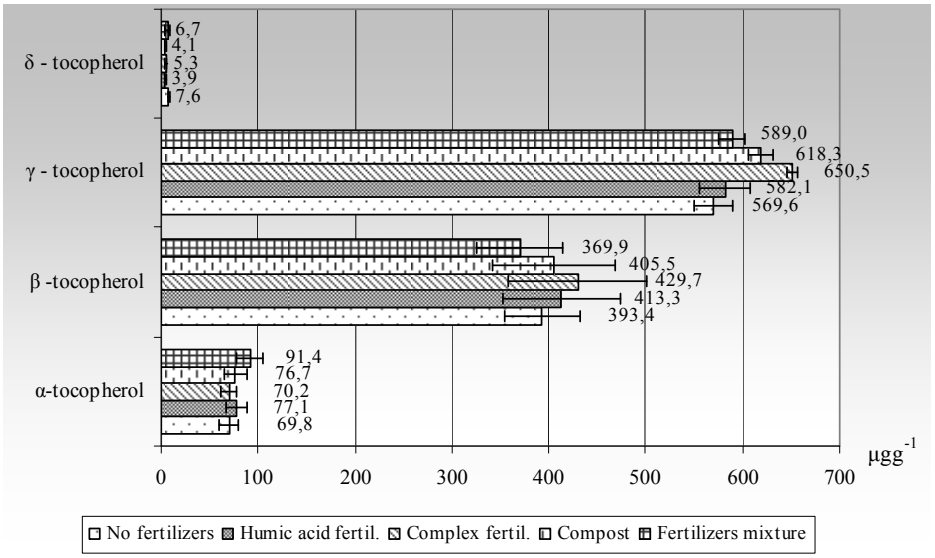

Fig. 6. Influence of fertilizers on tocopherol isomers content in oil pumpkin seeds oil [µg g^{-1}], LUA (means of 2005–2006)

Results obtained indicate that the applied fertilizers have no essential effect on tocopherol isomers content in oil pumpkin seeds and oil (Fig. 4 and Fig. 6).

Higher amount of δ- and γ-tocopherol isomers accumulates in the seeds when the raw material has been fertilized with the complex fertilizers, and of α-tocopherol – in the case of the humic acid fertilizers application. It has been established that, in comparison with the

control, the synthesis of β-tocopherols in pumpkin seeds, used for oil production, is slightly suppressed by the applied fertilizers, what results in their lower final accumulation (Fig. 4).

Analysis of different vitamin E isomers content reveals the predominance of γ-tocopherol, irrespective of cultivar and fertilization. It has been found that the amount of total vitamin E isomers in pumpkin oil ranged from 905 to 1336 μg g^{-1}, and α-, β- and γ- isomers of tocopherol dominated. In the oil pressed from the seeds of cv. 'Miranda' and 'Herakles', γ-tocopherol accumulated essentially (638 μg g^{-1} and 608 μg g^{-1}, respectively). β-tocopherol content reached 578 μg g^{-1} in oil of 'Miranda' seeds. The amount of α-tocopherol in pumpkin oil depended upon cultivar. The largest amount was found in 'Miranda' seeds (111.7 μg g^{-1}), and the smallest one – in 'Golosemiannaja' seeds (29.7 μg g^{-1}) (Fig. 5).

Data obtained showed that the applied fertilizers had no significant effect on tocopherol isomers content in pumpkin seeds oil (Fig. 6). Higher amount of β- and γ- isomers of tocopherol accumulated in the oil when the raw material had been fertilized with complex fertilizers, and of α-tocopherol – in the case of mixed fertilizers application. It has been established that, in comparison with the control, synthesis of δ-tocopherol in pumpkin seeds, used for oil production, is slightly suppressed by the applied fertilizers, what results in lower accumulation of this compound (Fig. 6).

Moderately strong and positive relationship has been found between fat-soluble isomers of α-, β-, γ- and δ-tocopherols and total fats content in pumpkin seeds ($r = 0.57$; $r = 0.61$; $r = 0.58$ and $r = 0.40$, respectively).

Conclusions

The γ-tocopherol was predominant isomer of vitamin E in oil pumpkin seeds and oil. The biggest quantity of γ-tocopherol was been found in 'Miranda' and 'Golosemiannaja' seeds, and also in 'Miranda' and 'Herakles' oil, but α-tocopherol isomer – in 'Miranda' seeds and oil. All applied fertilizers have no significant effect on tocopherol isomers content in pumpkin seeds and oil.

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3

OCCURRENCE OF PHTHALATES IN SOIL AND AGRICULTURAL PLANTS

Introduction

Phthalic acid esters (PAE) are widely extended organic compounds. They are used as plasticizer compounds in plastic materials, for example in plastic bottles, glues, paints, toys or PVC. Because of their unstability in plastic materials they are loosen to the environment. Due to their toxigenic and mutagenic properties, six PAE compounds are classified as priority pollutants [Cai et al., 2008].

Common commercial phthalates are liquids at ambient temperatures. Water solubility is a very important property which influences the biodegradation and bioaccumulation in some matters. Lipophilic character of PAE is the cause of their accumulation in the fat matters more than in the other matters.

Di-*n*-butyl phthalate (DBP) and di(2-ethylhexyl) phthalate (DEHP) are the most used.

Many studies have observed PAE contamination in soil, water and sediment [Michael et al., 1984; Vikelsøe et al., 2002]. As fertilizer is applied sewage sludge which can contain some of environmental pollutants, including PAEs. Periodic land application of sewage sludge may cause accumulation PAEs in the soil and in plants [Cai et al., 2008]. Consequently, these contaminants are came into food chain. The higher concentration of PAEs have been measured in the neighbourhood of phthalate-emitting plants [Müller & Kördel, 1993].

Nutrients and contaminants contained in soil solution are uptake by root system of the plants. The uptake of anthropogenic organic compounds by roots was occured by diffusive exchange between soil and roots in water and air pores, transfer into roots with the transpiration or assimilation steam [Trapp and McFarlane, 1995]. The phthalates are adsorbed on the soil sediments, which play important role in the uptake by plant. DEHP is more strongly adsorption on the soil sediment than DBP, thus the uptake of DEHP is much more lower [Schmitzer et al., 1988]. The uptake of DBP and DEHP by plants is species-specified [Aranda et al., 1989].

Microbial degradation is believed to be one of the major processes that remediate pollutan-contaminated soil [Chang et al., 2009]. The biodegradation of PAE in sediment, sludge and soil has been documented [Wang et al., 1996; Yuan et al., 2002]. If the particels are too large, specific surface area is smaller, and the number of potential sites for microbial activity is reduced [Delhomenie et al., 2002].

The aim of this study was to monitor occurrence and fate of DBP and DEHP in the environment. The samples of soil, plants (above-ground and below-ground parts of plants) were investigated.

Materials and methods

Chemicals

The standards of phthalic acid esters (DBP and DEHP), were 99% analytical standard grade (Sigma Aldrich, USA). The solvents for the extraction were obtained from Neratovice (Czech Republic) and solvent for HPLC/UV was obteined from Sigma Aldrich (USA).

Stock solutions of PAEs were prepared in acetonitrile in concentrations from 1,0 µg DBP/ml to 25,0 µg DBP/ml and 1,02 µg DEHP/ml to 25,5 µg DEHP/ml.

Sampling

Three fields were selected in the Middle Moravia in the Czech republic for our experiment. The samples of soil were collected before the sowing by sounding stick, from the 0–30 cm depth. *Triticum aestivum* was cultivated on the Location 1 and 2, and *Brassica napus* was cultivated on the Location 3. Every sample was compounded from six partial samples.

Samples of plants were removed in first phase of growth. Six group of small p were collected from location 1 and 2. From the location 3 six plants were collected.

Preparation and determination

Every sample of plant was washed and storaged at -18°C. All samples were dried by lyophilisation.

The extraction was performed by the method, which is described in the study of Jarošová (2004). The extraction were performed by mixture *n*-hexane and aceton 1:1 in three steps. Coextracts were separated by concentrated sulphuric acid.

The determination of DBP and DEHP were carried out by high performance liquid chromatography (HPLC) with UV detection at the wave length 224 nm. The column C18, particles size 5 µm (150 mm x 4,6 mm, Cogent) was used for separation. The mixture of acetonitrile and water in ratio 99:1 was used as mobile phase in HPLC/UV.

Results and discussion

The retention times of both of PAEs detected by HPLC can be determined from the chromatogram obteined (Fig. 1). DBP was detected in the third minute and DEHP in the seventh minute.

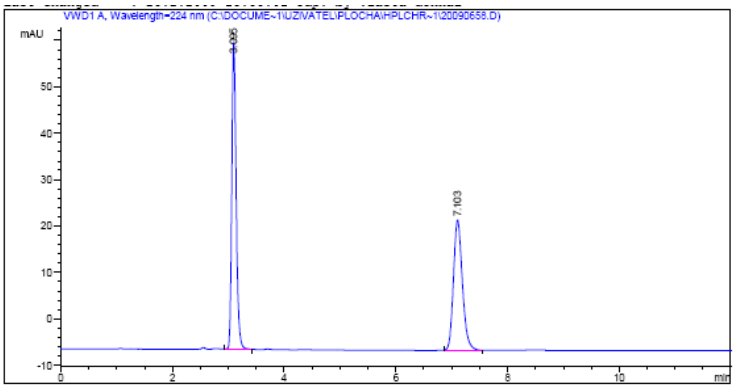

Fig. 1. Chromatogram with retention times of DBP and DEHP standards

Table 1 shows the results of occurrence of PAE in samples of soil from three location. The detection limit was determined 0,03 mg.kg^{-1}. The results showed that DEHP was not detected in any samples of soil, because the concentrations were below the detection limit (Fig. 2). These results we can partly attribute to the higher adsorbtion of DEHP on the soil particles.

Table 1

DBP and DEHP concentration in samples of soil (mg.kg^{-1} dry matter (DM))

	DBP	DEHP
Location 1	0,46	< 0,03
Location 2	0,55	< 0,03
Location 3	0,84	< 0,03

Several studies observed the higher concentration of PAE with increasing land application of manure. On the other site, in study of Petersen [2003] was observed no significantly correlation between DEHP concentrations in barley and increasing amounts of sewage sludge applied.

Vikelsøe [2002] have observed a various contents of phthalates in different depth of soil. In the clay soil DEHP maximum were occurred at a depth of 10–20 cm. The changes in the soil texture and dispersion processes may also be influenced by high concentration of DEHP in the clay layer [Kuhnt, 1993].

Metabolic breakdown of phthalates by microorganisms is considered to be one of the major process of environmental degradation. The availability of oxygen appeared to be a major regulator of DEHP degradation in soil and activated sludge [Roslev et al., 2007]. The degradation rates and half-lives of PAEs are strongly correlated with the alkyl chain length. PAEs with shorter alkyl chains were degraded more quickly [Wang et al., 2004].

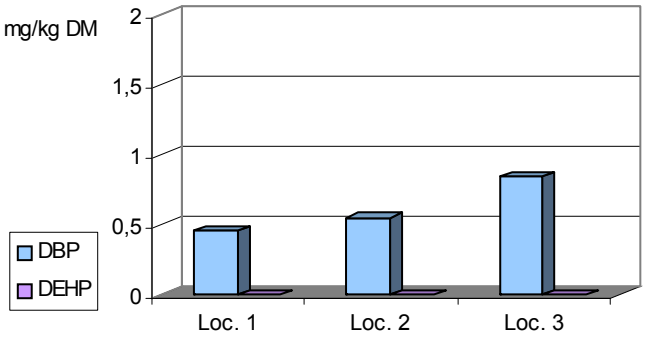

Fig. 2. The mean concentration of DBP and DEHP in soil [mg.kg^{-1} dry matter (DM)]

The mean concentrations of PAEs in agricultural plants are presented in Table 2.

In all samples of plants were found DBP concentrations significantly higher than DEHP.The amount of DEHP was in range from 0,12 to 2,27 mg.kg^{-1} DM and DBP from 3,5 to 14,26 mg.kg^{-1} DM.

PAEs, especially DEHP may be incorporated into soil humic substances, hence their bioavailability for the plant is significantly lower [Kottler and Alexandr, 2001].

Table 2

The concentrations of PAE in agricultural plants [mg.kg^{-1} DM]

		DBP	DEHP	Σ DBP + DEHP
Above-ground parts of plants	Loc. 1	7,38	0,53	7,91
Below-ground parts of plants		13,47	1,37	14,84
Above-ground parts of plants	Loc. 2	8,84	2,27	11,11
Below-ground parts of plants		14,26	0,68	14,94
Above-ground parts of plants	Loc. 3	3,5	0,42	3,92
Below-ground parts of plants		1,77	0,12	1,89

In study of Cai et al., [2008] the higher mean concentration of DEHP were observed in the shoots than in the roots of *Raphanus sativus*. About DBP, the results were not so significant. On the other hand, Yin et al., [2003] was observed higher amounts of DBP in the shoots than in the roots of capsicum, while, DEHP was not detected in any samples. The difference in plant uptake of DBP and DEHP might be caused their difference adsorption in soil and aqueous solubility. According to data, soil adsorption coefficients of DBP is 2144 and DEHP 67040, and their aqueous solubility are 11 and 0,4 mg.l^{-1} [Wang et al., 1996; Ye et al., 1993].

As evidence by Fig. 3–5, are significantly difference in the uptake of PAE by the various plant species. The results show, that *Brassica napus* uptake much more lower amounts of both of PAE than *Triticum aestivum*.

The other sources of phthalates were atmospheric deposition, manure or water, evidently.

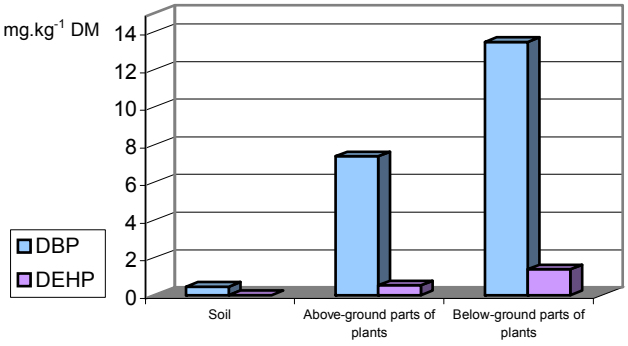

Fig. 3. DBP and DEHP concentration in soil and plants from Location 1 [mg.kg^{-1} DM]

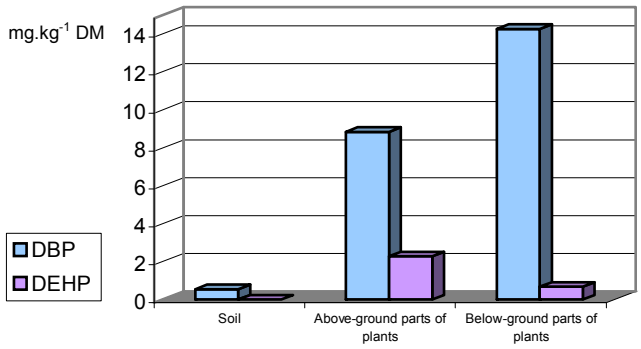

Fig. 4 DBP and DEHP concentration in soil and plant from Location 2 [mg.kg^{-1} DM]

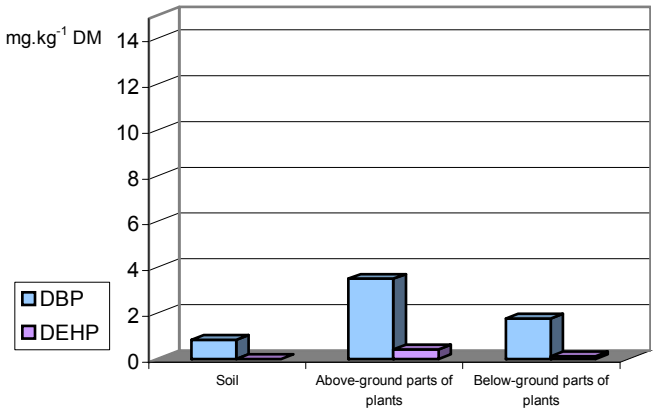

Fig. 5 DBP and DEHP concentration in soil and plant from Location 3 [mg.kg^{-1} DM]

Kato et al., [1980] reported that DEHP was not found in *Brassica rapa* grown in DEHP-contaminated soil. In several studies, have observed that uptake of DEHP by plants (*Lactuca sativa*, *Daucus carota*, *Capsicum annum*, barley) was rather low [Aranda et al., 1989; Schmitzer et al., 1988; Yin et al., 2003]. Our results are in agreement with these results.

In the case of wheat the sum of both of phthalates was higher in below-ground parts of plants than above-ground parts of plants (Fig. 6). The transport of pollutants in the soil-plant system is influenced by shape, length and area of the root. The increasing concentration of DBP may caused the reducing of biomass of root and shoot [Dueck et al., 2003]. The DBP concentations in tomato shoots and roots were significantly lower than those of DEHP [Sablayrolles et al., 2005]. Some studies have indicated that accumulation of organic contaminants in plants is species-specific [Parrish et al., 2006].

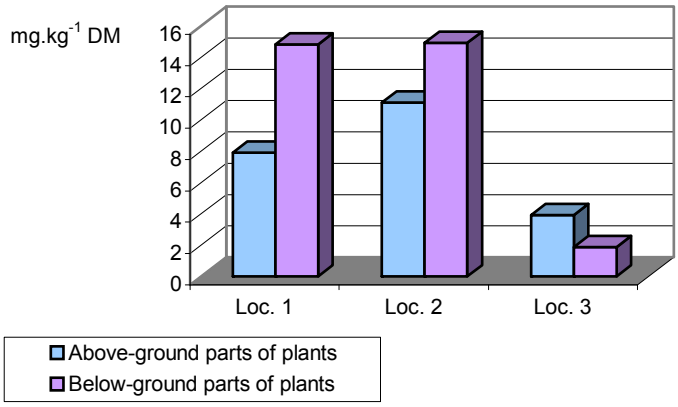

Fig. 6. The sum of DBP and DEHP concentrations in above-ground and below-ground parts of plants from each location [mg.kg^{-1} DM]

Conclusion

The phtalic acid esters were detected by high performance liquid chromatography with UV detection. DEHP was not detected in any samples of the soil. DBP and DEHP were observed in all samples of the plants. The mean concentrations of DBP in plants were detected from 3,5 to 14,26 mg.kg^{-1} dry matter. The concentration of DBP were detected lower in above-ground parts of plants than in below-ground parts of plants on the location 1 and 2. In the case of *Brassica napus,* the amount of phthalates was higher in above-ground parts of plants. The concentrations of DEHP were much more lower than concentration of DBP in all the samples of plants.

Acknowledgments

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4

FRESH WATER FISH AS A SOURCE OF PHTHALATES

Introduction

Phthalate esters are widely used industrial chemicals all over the world, not only as plasticisers but also as additives in industrial products, including food and personal care products [Petersen et al., 2000]. Among the most important phthalates belong dibutyl phthalate and di-2-ethylhexyl phthalate. Phthalic acid esters are found in huge range consumer products including floor and wall covering, furnishing, toys, car interior, clothing etc [Afshari et al., 2004, Schettler, 2006]. Higher molecular weight phthalate esters (for instance DEHP) act as an additive which imparts flexibility in vinyl products. Di-2-ethylhexyl phthalate is the dominant vinyl plasticizer. Lower molecular weight phthalates such as dibutyl phthalate have a very broad use which includes consumer products and pharmaceuticals.

Phthalate esters are not bound to the polymer with covalent chemical bonds and are therefore able to migrate to the surface of the polymer matrix where they may be lost by variety of physical processes. Because of their wide spread they are ubiquitous environmental contaminants.

Decomposing pattern depends on the length of side chain. The main metabolites of long chain phthalates are the oxidized metabolites [Wittassek and Angerer, 2008]. Short branched diesters are mainly excreted in urine as its monoester phthalates [Frederiksen et al., 2007].

The route of exposure that results in the most efficient absorption of phthalate esters is ingestion. The primary source of exposure is thought to be food. Minor levels of low- and high- molecular weigh phthalates can migrate into food from packaging or inks. In Europe, most of the food in contact with plastic contain DEHP and DBP. These are also found in common food products, such as cereals, bread, bis-cuits, cakes, nuts, spices, fat and oil in amounts up to about 10 mg/kg [Wormuth et al., 2006]. Recent studies have shown that infants are exposed to phthalates and their metabolites through breast milk, infant formulae and baby food [Schettler, 2006].

In general, phthalates show little acute toxicity. Acute lethal oral dose LD_{50} for DEHP is > 30,000 $mg.kg^{-1}$ bw and LD_{50} for DBP is > 8,000 $mg.kg^{-1}$ bw, both for rats. These data demonstrate that phthalates are relatively non-toxic following acute exposure. In animals additive effects of the phthalates have been clearly shown [Borch et al., 2004].

Effects of phthalates occur primarily in the liver or in the kidneys. High molecular weight phthalate dieters are the best inducers of metabolic changes among phthalates [Bentley et al., 1993]. Di(2-ethylhexyl)phthalate (DEHP) is a reproductive and developmental toxicant in animals and a suspected endocrine modulator in humans [Sharpe and Irvine, 2004, Lottrup et al., 2006]. Studies with DEHP have demonstrated that short-term exposure

does not cause long-term effects in the liver or testes. An epidemiologic study has shown that DEHP in house dust is associated with allergic asthma in children [Bornehag et al., 2004, Jaakkola and Knight, 2008]. Maternal exposure to DEHP during neonatal periods can accelerate atopic dermatitis in male offspring [Rie et al., 2008]. Becker et al., [2004] found that the oxidative metabolism of DEHP is dependent on age. In the children the ratios of the oxidized DEHP metabolites to MEHP generally increased with decreasing age. Age-dependent metabolism of phthalates may also have relevance to health: the oxidation products are longer in the human body than the simple monoesters, they might be more toxic [Stroheker et al., 2005].

Material and Method

The major problem in phthalate analysis is the risk of contamination, resulting in over-estimated concentrations. Due to the fact that phthalates are widely used, they are present in air, water, organic solvents, plastics and adsorbed on glass or other materials. Contamination can occur in every stage of the whole analytical procedure including sampling, sample preparation (extraction, clean-up) and chromatographic analysis. As far as possible, plastic materials should be removed from the laboratory. We did not use any plastic equipment and cleaned glass containers were rinsed with organic solvents to avoid contamination problem. Containers should not be left open, since they can adsorb phthalates from laboratory air onto the wall surface.

There were used 20 carps (*Cyprinus carpio*) in our work. Carps came from unnamed south Moravian pond that contains water from the river Dyje. The pond is used for breeding of carps and other fishes. Carps were fished out in the beginning of November 2008. 10 carps were analysed as soon as they were fished out. The second ten of fishes was storage in cement concrete cistern for a month.

Fish samples (skin and muscle) were first homogenized and then freeze-dried. We used liquid-liquid extraction and every sample was extracted three times (1hour-0,5 hour-0,5 hour) with using apolar mixture aceton : hexan (1:1). After extraction, the organic phase was concentrated by rotary evaporator and dried under nitrogen. Further, second clean-up step was performed due to co-extraction of fat. Since lipids have a polarity similar to the polarity of phthalates, it is necessary to remove them by a clean-up method based on size exclusion chromatography – gel permeation chromatography (GPC). The separation was achieved on the Bio-beads S-X3 column with a mobile phase of mixture of dichlormethan : cyklohexan (1:1). The mobile phase flow rate was $1mL.min^{-1}$. Injection of 1mL of sample was performed. Injected sample was composed of 0,5 gram of extract dissolved in the mobile phase. The DEHP peak elutes on the tail of the liquid peak. The fraction between 18 and 23 min was collected and concentrated. After evaporation of solvents out of sample, the extracts were purified three times by concentrated sulphur acid. Purified extracts were evaporated again under nitrogen and then they were dissolved in acetonitrile. The mixtures were homogenized by vortex agitation. Moreover, the analyses were performed by high pressure liquid chromatography (HPLC) on Separon SGX C 18 column with UV detection at 224 nm. Mobile phase was a mixture of acetonitrile : distilled water (9:1). The mobile phase flow rate was $1mL.min^{-1}$.

Area peak measurement with using Agilen ChemStation software for LC and LC/MS systems was performed for quantification of PAEs in the sample.

Results

Table 1

Concentrations of phthalates in $mg.kg^{-1}$ wet weight before winter storage

Sample	DBP	DEHP	∑ DBP + DEHP
1	1,00	1,73	2,73
2	1,65	1,66	3,31
3	0,94	1,95	2,89
4	0,96	2,48	3,44
5	0,95	2,81	3,76
6	0,66	2,80	3,46
7	1,40	3,03	4,42
8	1,29	1,97	3,26
9	1,09	2,44	3,53
10	1,42	3,04	4,46
Ø	**1,14**	**2,39**	**3,53**

Average concentration of DBP was 1,14 $mg.kg^{-1}$, DEHP 2,39 $mg.kg^{-1}$ and ∑DBP + DEHP 3,53 $mg.kg^{-1}$ fresh weight of carp before winter storage.

Table 2

Concentrations of phthalates in $mg.kg^{-1}$ wet weight after winter storage

Sample	DBP	DEHP	∑ DBP + DEHP
1	1,05	0,01	1,06
2	0,94	0,16	1,10
3	0,84	0,00	0,84
4	0,96	0,18	1,14
5	1,32	0,05	1,38
6	1,04	0,08	1,12
7	2,08	0,33	2,41
8	1,40	0,32	1,72
9	1,76	0,61	2,37
10	1,59	0,25	1,84
Ø	1,30	0,20	1,50

Average concentration of DBP was 1,30 $mg.kg^{-1}$, DEHP 0,20 $mg.kg^{-1}$ and ∑DBP + DEHP 1,50 $mg.kg^{-1}$ fresh weight of carp after winter storage.

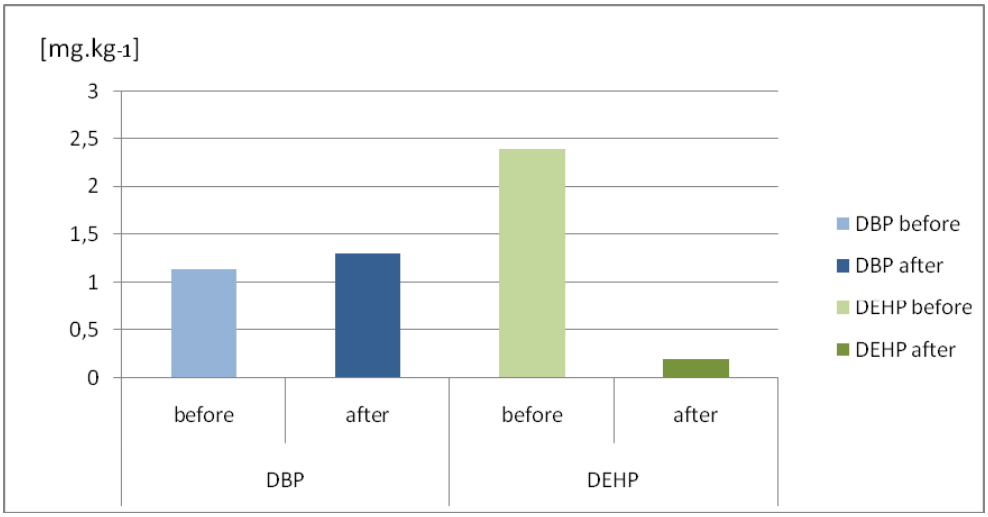

Fig. 1. Comparison of average concentrations of both phthalate before and after winter storage

There was approximately same level of DBP before and after winter storage of carps.

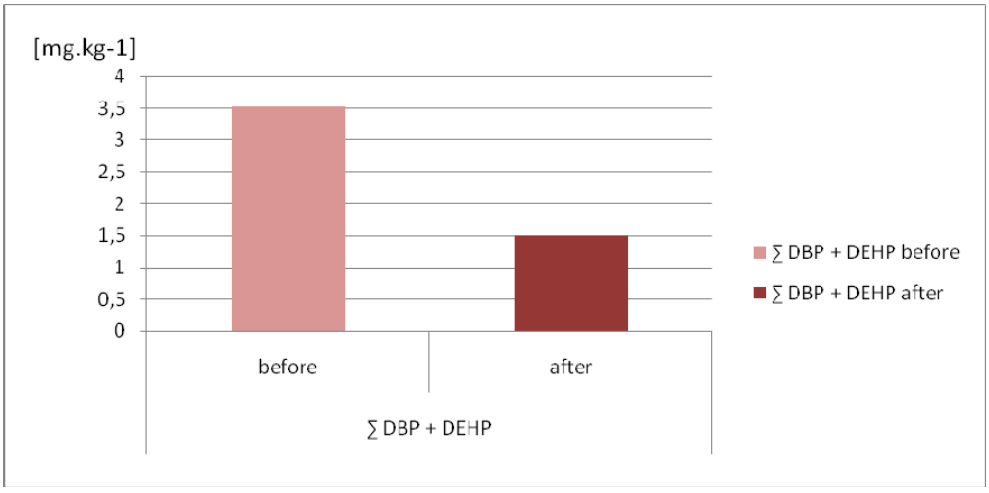

Fig. 2. Comparison of ∑DBP + DEHP before and after winter storage

There was significant decrease of ∑DBP + DEHP after winter storage.

Conclusions

We observed impact of winter storage on content of phthalate esters in carps tissues. Carp (*Cyprinus* carpio) is a dominant fish of freshwater fish market in the Czech Republic. Carp is a major part of Czech Christmas dinner.

Phthalates are lipophilic compounds and so they are accumulated in the adipose tissue. Wormuth at al. [2006] found the highest phthalate esters concentration of food in edible oils and fat. Carp's skin contains high volume of fat, consequently skin is the main source of phthalates in fishes. Huang et al., [2008] investigated phthalate compounds in sediments and fishes in 17 Taiwan's rivers. Their data suggested that DEHP level in river sediments were influenced by water quality parameters due to their effects on the biodegradation processes, and that the DEHP level in fish was affected by fish habitat and physiochemical properties of polluted contaminants.

In our work, carps without winter storage lived in natural south Moravian pond. Their bodies contained two-fold higher volume of di-2-ethylhexyl phthalate (DEHP) than di-n-butyl phthalate (DBP). DEHP is more spread in the environment due to its wide range of applications.

Winter storage is temporary storage of fished out fishes till sale time. After winter storage there was significant decrease (twelve-fold) of DEHP content in carp's tissues. Water solubility is an extremely important property that influences the biodegradation and bioaccumulation potential of a chemical, as well as aquatic toxicity [Staples et al., 1997]. DEHP is water insoluble and adsorbed to solid and organic compounds in water. Carps look for their feed in sludge and are often in contact with it. Consequently DEHP contamination of their bodies is higher. Because they live near to sludge their can taste as mud. After winter storage carps lost "mud smell" in running water. As we found, winter storage has next additional advantage. Carps without contact with pond mud, main source of phthalate esters in water, metabolize during winter storage toxic environmental contaminants and its concentration decrease (more than two-fold in our work). During winter storage there are only little important sources of phthalates from water and air.

DBP is more water soluble then DEHP, due to its physical-chemical properties and is little spread in the environment.

Our findings are important from the food safety point of view. According to Oehlmann et al., (2008) there are no reliable long-term studies below the apparent water solubility of DEHP indicating effects on organisms exposed to DEHP in water.

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5

RELATIONSHIP BETWEEN THE LEVEL OF NITROGEN CROP FERTILZATION, NITRATES CONTENT IN CATTLE FEED AND OBTAINED MILK

Introduction

The yield and characteristic of agricultural crop depend on many different factors. One of these is mineral fertilization, especially nitrogen. Although substantial, it can be a source of risk.

The objective of this study is to control the content of mineral nitrogen in the soil, in order to optimize nitrogen fertilization, and to reduce the content of NO_3 in plant, milk and meat.

Material and Methods

Samples of soil, cattle feed and milk were taken from a dairy farm in the Vojvodina Province. The available nitrogen content was determined in the Spring by the method of Scharp and Werthman. The obtained results were used to plan nitrogen fertilization.

Cattle feed was preparing 24 hours before milk sampling. The samples were transported to the laboratory in portable refrigerators, at 2–6°C. Ground samples of feed were extracted in distilled water for 10 min. Nitrate content in milk was monitored individually for each dairy cow. An aliquot of the sample was treated with hydrogen peroxide and evaporated in a sand bath at 100–120°C. After evaporation and cooling, the samples were treated with disulfonic acid and quantitatively transferred into a volumetric flask. Ammonium hydroxide was added to the flask for stain development. After staining completion, reading was performed at the wavelength of 420 μm. Nitrates were expressed in ppm. Nitrate content in water supplied to the test animals was not monitored.

Results and discussion

At the first part of the research, nitrate contents were determined in milk and cattle feed. The obtained results are shown in Table 1.

Table 1

Nitrate contents in cattle feed and milk [ppm]

Date of sampling	Cattle feed			Milk (per group of cows)							Average
	Hay	Silage	Conc.	1	2	3	4	5	6	7	
9 Jan. 1998	750	425	75	14.6	22.0	20.1	–	–	–	–	18.9
30 Jan. 1998	1175	750	200	32.0	24.2	31.0	32.0	38.5	48.0	–	34.3
16 Feb. 1998	600	19	–	22.5	13.2	31.2	29.0	29.0	25.7	24.0	24.9
23 Feb. 1998	250	–	–	13.2	7.0	11.7	16.0	21.7	12.5	19.2	14.3
7 Mar. 1998	700	400	175	29.0	22.5	27.2	32.0	–	–	–	27.7
21 Mar. 1998	1700	1150	825	27.2	21.7	22.5	29.0	32.0	32.0	–	27.4
4 Apr. 1998	–	150	–	8.5	8.5	7.5	–	–	–	–	8.2
Average				22.2	16.9	22.7	25.5	27.4	26.3	21.6	

Lowest nitrate contents were found in concentrated feed, from 0 to 825 ppm. Much higher contents were found in silage (from 0 to 1150 ppm), and highest in alfalfa hay (from 0 to 1700 ppm). Nitrate content in milk depended directly on nitrate content in cattle feed.

The lowest nitrate content in cattle feed (150 ppm on 4 May 1998) brought about lowest nitrate contents in milk, from 7.5 to 8.5 ppm. On 16 March 1998, milk samples had a high average nitrate content (24.9 ppm), while nitrates were low in feed (600 ppm in hay, 19 ppm in silage). This difference was probably due to high amounts of nitrates in the water provided for dairy cattle. At the other sampling dates, the total nitrate content in feed was increased, ranging from 1250 to 1275 ppm. At these dates, the nitrate content in milk was from 18.9 to 27.7 ppm. Further increases of nitrates in the feed, to 2125 ppm, led to a significant increase of the average nitrate content in milk, to 34.3 ppm (the actual values ranging from 24.2 to 48.0 ppm).

In the second part of the research, nitrates were monitored in the soil, cattle feed and milk. These results are shown in Tables 2 and 3.

Table 2 shows nitrate contents along the soil profile in the corn and alfalfa fields analyzed in the Spring of 1998.

Table 2

Distribution of nitrates along soil profile [$kgNha^{-1}$]

	0–30 cm	30–60 cm	60–90 cm	90–120 cm	Σ	Yield, tha^{-1}
Corn 1	77.68	89.50	54.60	71.72	293.56	9.8
Corn 2	51.70	45.96	21.10	9.30	128.06	10.2
Corn 3	49.21	76.60	32.58	14.76	173.14	10.4
Alfalfa 1	54.76	42.96	18.54	12.56	128.84	10.0
Alfalfa 2	52.32	74.61	38.58	16.76	182.27	9.3
Alfalfa 3	47.25	81.55	55.79	26.24	210.83	8.1

Table 3

Nitrate contents in cattle feed and milk [ppm]

Date of sampling	Cattle feed			Milk (per group of cows)			Average
	Hay	Silage	Conc.	1	2	3	
9 Sep. 1998	708	–	307	5.1	27.5	22.5	18.4
16 Sep. 1998	643	250	75	5.4	16.0	15.0	12.1
23 Sep. 1998	1217	–	250	5.8	22.5	22.5	16.9
30 Sep. 1998	550	19	–	4.8	9.4	7.0	7.1
6 Oct. 1998	762	150	75	5.2	15.2	18.4	12.9
13 Oct. 1998	610	170	60	4.9	15.7	20.0	13.5
20 Oct. 1998	820	156	80	5.8	17.2	18.6	13.9
27 Oct. 1998	1108	250	170	6.1	26.5	27.2	20.6
4 Nov. 1998	1260	250	190	5.8	27.0	28.6	20.5
11 Nov. 1998	480	50	–	4.2	14.8	16.2	11.7
18 Nov. 1998	510	60	–	3.8	15.1	17.0	12.1

The calculation of fertilizers dose required for corn yield of 11 tha^{-1} indicated that corn plot 1 had excess nitrogen in the amount of 155 $kgha^{-1}$. The surplus N in corn plot 3 was 34 kg ha^{-1}, while corn plot 2 required additional 31 kg N ha^{-1}.

In 1998, yields of corn and alfalfa ranged from 9.8 to 10.2 tha^{-1} and from 8.1 to 10 tha^{-1}, respectively, which confirmed the observation that the soil nitrogen was in excess.

Nitrate content in alfalfa hay was directly correlated with nitrate content in the soil.

Highest nitrate contents in hay were registered in alfalfa plot 3, 1217, 1108 and 1260 ppm. Alfalfa plot 2 had lower nitrate contents in hay, 708, 643, 762 and 820 ppm. The latter plot had 182 kg $N\text{-}NO_3$ ha^{-1} in the Spring

The lowest nitrate content of in the soil (129 kg N^-NO_3 ha^{-1}) and hay was in alfalfa plot 1. Nitrate contents in hay were 550, 610, 480 and 510 ppm. It should be noted here that this was alfalfa in the first year of use.

Nitrate content in cornstalks and corn grain was also directly dependent nitrate level in the soil. In corn plot 1 there was an excess of 155 kg N^-NO_3 ha^{-1} in the spring. Nitrate contents in cornstalks and grain from that plot were 250 and 170–307 ppm, respectively. In corn plot 3, the respective NO_3 contents were lower, 156–170 and 60–80 ppm. The lowest nitrate contents were found in corn plot 2, 50–60 and 0 ppm, respectively.

The highest nitrate contents in milk were registered on 27 October and November 1998, in the period when the dairy cows received feed rich in nitrates, which originated from over-fertilized plots (alfalfa plot 3 and corn plot 1). On the two sampling dates, the nitrate contents in alfalfa hay, cornstalks and grain were 1108 and 1260 ppm, 250 and 250 ppm, and 170 and 190 ppm, respectively. The average nitrate contents in milk were 20.5 and 20.6 ppm, respectively, the actual values ranging from 5.8 to 28.6 ppm. Lower nitrate contents in milk were registered on 16 September, 6 October and 20 October 1998 (12.1, 12.9 and 13.9, respectively), when the cows received hay from alfalfa plot 2 with medium nitrate levels (643, 762 and 820 ppm, respectively), cornstalks from corn plots 1 and 3 with high

nitrate levels (250, 150 and 156 ppm, respectively) and grain from corn plot 3 with low nitrate levels (75, 75 and 80 ppm, respectively). On 11 and 18 November 1998, nitrate contents in milk were slightly lower (11.7 and 12.1 ppm, respectively). On those dates the cows received feed from the alfalfa plot with the lowest nitrate content in the soil (129 kg NO_3 ha^{-1}) and with cornstalks and grain from the corn plot also with the lowest nitrate content in the soil (128 kg NO_3 ha^{-1}). The same feed was used on 30 September 1998, when the nitrate content in milk was only 7.1 ppm. In this case, nitrate level in cornstalks was 30-40 ppm less compared with the previous case, which definitely affected nitrate content in milk. In our opinion, there was another reason hat affected the NO_3 content in milk, and this is the nitrate content in water supplied to the test animals. It is important for this report to take notice of the fact that animal 1 has consistently manifested a lower nitrate content in milk than the other two animals, which in their turn had similar nitrate levels in milk.

A major shortcoming of this study is that we did not monitor the content of nitrates in the water supplied to the test animals. Alternatively, we could have monitored the content of nitrates 36 hours before taking milk samples. This oversight should be corrected in further research.

The results of this study, similar to the results of Gorlitz [1984] and Marinković et al. [1982/2, 2008] confirm that corn fertilization may be scheduled by the N-min method. Increased nitrogen content in the soil directly increases the content of NO_3 in corn plants, which was evident in this study as well as those of Jelenić [1984], Mattas et al. [1965], etc.

Conclusion

Based on the results obtained in the given agro-ecological conditions, the following can be concluded.

Nitrogen was not a limiting factor in the productions of corn (9.8 to 10.2 tha^{-1}) and alfalfa hay (8 do10 tha^{-1}).

Highest nitrate contents were registered in alfalfa hay, from 0 to 1700 ppm, somewhat lower in silage, from 0 to 1150 ppm, and considerably lower in cornstalks, from 19 to 250 ppm, and corn grain, from 0 to 307 ppm.

Due to differences in composition, nitrates in concentrated feed ranged from 0 to 825 ppm.

Nitrate content in cattle feed depended on nitrate content in the soil.

At optimum nitrate contents in alfalfa and corn fields (129 and 128 kg NO_3 ha^{-1}, respectively) cattle feed had lowest nitrate contents (about 500 ppm in hay, around 55 ppm in cornstalks and 0 ppm in corn grain) while nitrates in milk ranged from 5.8 to 17.0 ppm.

At high nitrate contents in alfalfa and corn fields (211 and 294 kg NO_3 ha^{-1}, respectively) cattle feed had high nitrate contents (about 1200 ppm in hay, around 250 ppm in cornstalks and around 180 ppm in corn grain) while nitrates in milk ranged from 29.2 to 34.1 ppm.

High correlations existed between nitrate contents in the soil, cattle feed and milk.

The examined groups of animals as well as individual animals differed significantly in nitrate content, from 1.0 to 34.3 ppm.

The obtained results indicated clearly that high yields of agricultural crops may be achieved by cost-efficient application of nitrogen fertilizers. Cattle feed produced in that way contains much less nitrates and it bears corresponding effect on nitrates in milk.

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6

STUDY ON SLAUGHTER VALUE AND SOME MEAT QUALITY TRAITS IN PIGS OBTAINED FROM CROSSING NAIMA AND PBZ SOWS WITH DUROC BOARS

Introduction

Considerable improvement has been observed in Poland for many years now in terms of the quality of slaughter animals. This is manifested both in an increase in meatiness of fatteners and the proportions of the most valuable joints in the carcass as well as reduced carcass fatness [Grześkowiak et al., 2005]. The dynamic development of genetics and its use in animal breeding have resulted in the selection of pigs with the most desirable traits, at the simultaneous elimination of disadvantageous traits. In this context we also need to mention the role of fatteners of imported high meat producing lines introduced to commercial production. In the Polish raw material base we may observe an increasing proportion of pigs from hybrid lines.

The Duroc breed is a breed used commonly in commercial crossing. It is characterized by optimal meatiness and a relatively low proportion of quality defects. An additional advantage improving particularly sensory attributes is a higher content of intramuscular fat than those in other breeds [Grześkowiak 2002, Grześkowiak et al., 2003, Eckert 2000, Różycki 2003, Niemyjski 2007].

Pigs of Chinese breeds, characterized by hyperproloficacy and good carcass quality, were used in the creation of a maternal, hybrid Naima line, thanks to which sows with optimal maternal traits were selected [Niemyjski 2003].

The aim of the study was to compare slaughter value and meat quality of fatteners coming from mating of Duroc boars with sows of a native Polish White Landrace [Polska Biała Zwisłoucha – PBZ] and sows of a hybrid Naima line.

Material and methods

Analyses were conducted on 40 carcasses of fatteners coming from crossing of Duroc boars with Polish White Landrace sows (n = 12 carcasses) and a hybrid Naima line (n = 28 carcasses). All fatteners were produced in a selected producers' group, maintaining similar management conditions and identical feeding regimes with all-mash, coming from one feed producing plant. Fatteners, both gilts and boar piglets at a 1:1 ratio, after reaching a weight of approx. 115 kg were transported to an abattoir (transport at a distance of approx. 30 km) and slaughtered there, after 1 – 2 h rest, in accordance with the regulations binding in meat industry. In order to determine slaughter value of carcasses the following measurements

were taken: meat content in the carcass using an optical needle choirometer CGM [Borzuta et al., 2004], carcass weight on an overhead scale accurate to 100 g, tissue acidification at 45 min after stunning (pH_1) using a pH-meter by SYDEL with a combined stiletto electrode, as well as backfat thickness using an electronic nonius at 5 points, i.e. at the sacrum I, II, III, at the back and the shoulder. Half-carcasses after 24-h cooling were divided into primal cuts according to the industrial standard (PN-86-A/82002) in order to determine the weight and percentages of cuts. During cutting samples for further laboratory analyses were collected from the lumbar section of the *longissimus dorsi* muscle. These analyses included the determination of several meat quality attributes, such as pH_2 (24 h *post mortem*), water holding capacity, colour in the L*,a*,b* system, natural drip, weight loss during thermal processing, shear force, as well as the determination of marbling and basic chemical composition of meat. Samples of thermally processed meat and cured and smoked LD muscle were subjected to organoleptic analyses of aroma, tenderness, juiciness and flavour (PN-ISO 4121:1998).

Results of measurements were analysed statistically. The variance of means was determined by calculating standard deviation. Statistical significance of the effect of analyzed experimental factors on quality attributes was determined using Student's t-test [Stanisz, 1998].

Results and discussion

Fatteners of both groups were characterized by comparable meat content in the carcass, i.e. 56.5% for fatteners after a Polish White Landrace sow and 55% for fatteners from the group, where sows came from the Naima line (Tab. 1), with fatteners from the former group having a higher carcass weight. No carcasses with extremely PSE meat, with pH45 lower than 5.8, were found in either group [Borzuta 2003, Krzęcio et al., 2003]; however, pH45 values of fatteners after Polish White Landrace sows were significantly lower. Results of studies conducted by other authors indicate a relatively high percentage of PSE meat in Polish White Landrace pigs, which results from the breed-specific predisposition of these pigs connected with poorer adaptation to stress conditions [Różyczka et al.,1978, Michalski 1988, Grześkowiak 1995]. Backfat thickness did not differ statistically significantly between the analyzed groups.

It results from the percentages of primal cuts presented in Table 2 that fatteners coming after a Polish White Landrace dam were characterized by a significantly higher proportion of loin and ham.

As it results from Figure 1, which presents sensory examination scores of cooked meat and raw smoked sirloin, no significant differences were found between analyzed groups, except for the score for uniformity and desirability of colour for smoked sirloin, which turned out to be more advantageous in fatteners after a Naima dam.

Moreover, statistically significant differences were found between analyzed physicochemical traits of meat (Tab. 3). We need to stress here an inferior water holding capacity, increased drip loss during thermal processing as well as a higher water content and lower protein content in fatteners after Polish White Landrace dams.

Table 1

Mean results of slaughter value and carcass quality of investigated fatteners groups

Traits	PBZ x Duroc		Naima x Duroc	
	x	s	x	s
Carcass weight, kg	104,15	4,21	98,87	8,42
Meat content in carcass, %	56,5	2,34	55,0	3,38
Backfat thickness, mm				
over shoulder	38,7	2,45	40,40	7,31
on the back	24,30	3,16	24,60	5,60
on cross I	24,80	4,61	27,67	7,31
on cross II	16,80	3,01	17,67	6,51
on cross III	$21,60^{A}$	4,01	$27,03^{B}$	7,77
pH_{45}	$6,38^{A}$	0,26	$6,59^{B}$	0,22
EC_{24}	3,67	1,23	3,33	1,53

A,B difference significant at P<0,01 between groups

Table 2

Percentage share of primary cuts of carcasses in investigated groups

Primal cuts	PBZ x Duroc		Naima x Duroc	
	x	s	x	s
Neck	6,90	0,69	7,11	0,63
Loin	$10,36^{A}$	0,76	$9,51^{B}$	0,92
Ham without shank	$26,83^{A}$	1,31	$25,38^{B}$	1,04
Shoulder without shank	$13,24^{A}$	0,55	$14,22^{B}$	0,65
Backfat	5,66	0,79	5,36	0,86

A,B difference significant at P<0,01 between groups

Table 3

Meat quality charakteristic of fatteners from investigated groups

Traits	PBZ x Duroc		Naima x Duroc	
	x	S	x	s
WHC, %	$34,00^{A}$	1,85	$29,89^{B}$	2,94
Drip losses, %	3,27	1,19	3,33	1,64,
Cooking losses, %	$32,14^{A}$	2,95	$27,16^{B}$	5,12
Water content, %	$73,73^{A}$	0,53	$72,91^{B}$	0,95
Fat content, %	1,94	0,24	2,21	1,06
Total protein content, %	$23,16^{A}$	0,41	$23,71^{B}$	0,70
Colour, L	47,97	1,69	47,57	1,83

A,B difference significant at P<0,01 between groups

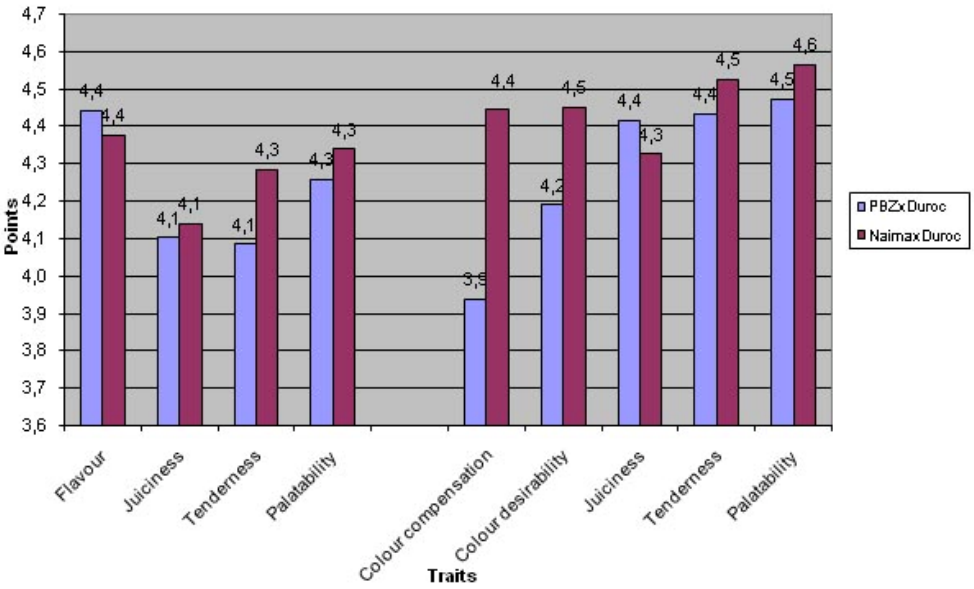

Fig. 1. Sensory properties (points) of cooked muscles LD and smoked loin

Conclusions

Based on the conducted analyses it was found that crosses coming after a Polish White Landrace dam and a Duroc sire were characterized by slightly better slaughter value traits than those after a Naima dam and a Duroc sire (slightly higher meatiness and carcass weight, a higher proportion of loin and ham). In turn, superior meat quality attributes were found in fatteners after a Naima dam. This pertains particularly to water holding capacity, thermal drip loss, lower water content, higher protein content and better uniformity and desirability of colour of raw sirloin. No statistically significant differences were found in sensory quality of cooked meat and smoked sirloin between both compared groups.

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7

THE EFFECT OF CONTENTS OF MYCOFLORA AND TRICHOTHECENES IN WHEAT GRAIN ON QUALITY OF ITS MILLING PRODUCTS

Introduction

The food pyramid, presenting the scheme of adequate human nutrition, is based on components, which are most important for the consumer both from the nutritional and health state point of view. It results from the analysis of the composition of daily food rations that human nutrition is based on processed cereal products. Experts on nutrition are of the opinion that daily consumption of processed cereals by healthy adults should be jointly 260 – 600 g, i.e. from 5 to 11 servings of cereal products, depending on the volume of daily energy requirement and eating habits. High requirement and consumption of this type of products enforces the necessity of their intensive control at each stage of production [Filtenborg et al., 1996]. Major pathogenic microorganisms affecting quality of cereal products include microscopic fungi. Toxic secondary metabolites of these fungi called mycotoxins [Clare & Sue, 2004], may have a significant effect on the quality of grain, milling products and as a result - that of the final product, i.e. bread. In infected kernels starch granules as well as reserve protein are damaged. This results in deterioration of grain quality. Fusarium infected grain has reduced weight by volume and 1000 kernel weight, as well as low glassiness [Bennet & Richard, 1996]. Flour produced from such grain has high ash content, considerably reduced protein content and gluten content reduced by half [Jackowiak et al., 2005; Muthomi et al., 2002]. Dough is moist, with viscous surface, while bread yield is very low, crumb is dark and of low elasticity. Microbiological contamination of cereals has a direct effect on purity of flour and other grain milling products, and thus also the quality of final products, mainly bread [Börjesson et al., 1990]. The numbers and type of microorganisms penetrating from grain to flour depend on the degree to which grain is cleaned prior to milling [Neuhof et al., 2008; Stuper & Perkowski 2008; Weidenborner et al., 2000]. To date several microbiological, enzymatic and chemical methods have been developed to determine contents of both mycoflora and its metabolites [Miedaner & Perkowski, 1996; Perkowski et al., 1990; Scott, 1984; Tkachuk et al., 1991]. This study is based on the method analyzing contents of ergosterol (ERG), which is a specific fungal marker [Tothill et all., 1992; Young, 1995; Zhao et al., 2005]. ERG is a component of the cell wall in moulds and is used to determine the content of mycoflora in tested plant material. In the presented paper the analysis of mycotoxin contents was also applied. Due to the fact that in the investigations the selected samples of wheat grain varied in their degrees of infestation and in their mycoflora the dominant species were fungi from genus *Fusarium*, we only analyzed concentrations of trichothecenes B.

The aim of this study was to analyze changes in concentrations of ERG and selected mycotoxins at each stage of bread production, starting from the parent material, i.e. grain of two wheat cv. (Torka and Griwa), through their milling products, until the final product, i.e. bread. Additionally basic characteristics of flour and bread, determining their technological quality, were determined.

Since cereal grain constitutes the staple raw material in the production of food for humans and feed for animals, the postulate of obtaining the lowest possible concentrations of ergosterol and mycotoxins in semi-processed products and final foodstuffs is fully justified. In view of the above it needs to be remembered that in the process of flour production a considerable decrease is observed in the concentration of ergosterol and mycotoxins.

Materials and methods

Experimental material comprised samples of wheat grain cv. Torka and Griwa, bred and harvested at the Experimental Station in Bałcyny, where conditions promoting growth of mycoflora were found, the samples being referred to in the further part of the paper as experimental samples. Moreover, the control for both cultivars was analyzed, which was characterized by a low concentration of ergosterol. The weight of samples was approx. 2000g. Samples were milled under laboratory conditions using a Brabender mill at the Faculty of Food and Nutrition Sciences, the Poznań University of Life Sciences. As a result of milling flour type 750 and bran were produced. The following parameters of flour were determined:

- Flour acidity assayed following Standard PN-60/A-74007
- Flour yield expressed in percent,
- Moisture content
- quality and amount of wet gluten determined following Standard PN-A-74043-2

Experimental bread was baked using the direct one-stage method. Examination of bread in a point scale was conducted after 24 h.

In grain and its milling products the ERG concentration is used as an indicator of mycoflora content and concentration of trichothecenes B.

Method of ergosterol determination

Samples were analyzed for the presence of ergosterol according to Perkowski et al. (2008). Briefly, samples of 100 mg were placed into 17 ml culture tubes, suspended in 1 ml of methanol, treated with 0.1 ml of 2 M aqueous NaOH, and sealed tightly. Then the culture tubes were placed within 250 ml plastic bottles, sealed tightly, and placed inside a microwave oven (Whirpool model AVM 401/WH) operating at 2450 MHz and 900 W maximum output. Samples were irradiated (370 W) for 20 s, after *c*. 5 min for additional 20 s and extracted with pentane (HPLC grade, Sigma-Aldrich, Steinheim, Germany) (3 x 4 ml) within the culture tubes. The combined pentane extracts were evaporated to dryness in a gentle stream of high purity nitrogen. Prior to analysis samples were dissolved in 4 ml of methanol, filtered through 13 mm syringe filters with 0.5 μm pore diameter (Fluoropore Membrane Filters), evaporated to dryness in a stream of nitrogen and dissolved in 1 ml of methanol. Prepared samples were analyzed by HPLC. Separation was run on a 150 mm length

x 3.9 mm diameter Nova Pak C-18, 4 μm particle size column and eluted with methanol/acetonitrile (90:10) at a flow rate of 0.6 ml/min. ERG was detected with a Waters 486 Tunable Absorbance Detector set at 282 nm. Estimation of ERG was performed by a comparison of peak areas with those of an external standard (> 95%, Aldrich, Milwaukee, USA) or by co-injection with a standard. Detection level was 0.01 mg/kg.

Method of trichothecene B determination

Samples of 10 g were milled using a WŻ-1 laboratory mill (the Research Institute of Baking Industry, Bydgoszcz). Samples, after being placed in 200 cm^3 Erlenmeyer flasks, were extracted using a 100 cm^3 acetonitryle–water mixture at 82 : 18 (v/v) by shaking for 15 min, and next being left overnight and repeated shaking for 15 min. Obtained extracts were filtered through Whatman no. 5 cellulose filters on Büchner funnels under reduced pressure.

Extracts were purified by extraction to the solid phase using columns packed with (5 cm^3) mixture of active carbon (Draco G 60, 100 mesh), celite (Celite 545) and neutral aluminium oxide (70-230 mesh) mixed at a weight ratio of 1:1:1.

Columns were prepared as follows: in 6 cm^3 polypropylene columns packed successively with glass wool, 0.25 g celite, 3 g mixture f active carbon, celite and neutral aluminium oxide, followed by glass wool. Such prepared columns were activated by rinsing with 15 cm^3 acetonitryle–water mixture (82 : 18 v/v) under reduced pressure at a flow rate of 1 cm^3/min. After flasks were emptied to columns, filtered extracts were introduced and next columns were washed with a 30 cm^3 acetonitryle–water mixture (82 : 18 v/v). Both combined filtrates were collected and evaporated to dryness using a vacuum evaporator (Büchi R-205). The residue was transferred quantitatively using ethyl acetate (2.5 cm^3) and 2 portions of 2.5 cm^3 each of a chloroform – acetonitryle mixture (4 : 1 v/v) to 8 cm^3 vials by evaporating on the on-going basis in a stream of nitrogen.

Trichothecenes B (deoxynivalenol – DON, 3-acetyldeoxynivalenol – 3-AcDON, 15-acetyldeoxynivalenol – 15-AcDON, nivalenol – NIV, fusarenone X – FUS-X) were analyzed as trimethylsilyl derivatives using an external standard. Trimethylsilyl derivatives were obtained in a reaction with a mixture of trimethylsilyl imidazole and trimethylchlorosilane (100 : 1 v/v, 100 μl) run in a 8 cm^3 vial at room temperature for 20 minutes. The reaction was stopped by adding to the reaction mixture the amount of 3 cm^3 distilled water and next 0.5 cm^3 isooctane was added, it was shaken in a shaker (MS 1, IKA Works, INC) and the isooctane layer was transferred to a tightly sealed vial. Chromatographic separation and the analysis of trichothecenes B were run using a gas chromatograph (Hewlett Packard 6890) coupled with a mass detector (Hewlett Packard 5972 A). The apparatus was equipped with an autosampler (HP 18593B) and a capillary column (HP-5MS, 0,25mm x 30 m). Samples of 1 μl were injected to an injector chamber at 280°C without stream division, at the separator temperature of 280°C. In the assay of trichothecenes B the initial oven temperature was 80°C and it was maintained for 1 minute. Next the temperature increased by 15°C/min to 200°C and it was maintained for 6 minutes. After that time the temperature increased by 10°C/min to 280°C and it remained at that level for 5 minutes. The total time of analysis was 28 minutes. In order to determine trichothecenes B the analysis of selected ions was conducted (SIM). These were: for DON ions 103 and 512; 3-AcDON 117 and

482; 15-AcDON 193 and 482; FUS 103 and 570; NIV 191 and 600, respectively. Retention time for these toxins was 19.53, 20.88, 21.07, 21.01 and 21.25 minutes. The flow rate for helium was 0.7 cm^3/min. In order to confirm the presence of assayed toxins in the sample an analysis was conducted over the entire range of weights (from 100 to 700 amu) yielding the mass spectrum, which was compared with an analogously obtained spectrum for the standard. This spectrum together with the comparisons of retention times for the analyzed compound with the standard constituted the basis for the identification of toxins. Apart from quality analysis the concentrations of analyzed toxins were determined by comparing relative heights of selected ions. Results were subjected to processing in the ChemStation program. Recovery rates for analyzed toxins were as follows: DON 84±3.8%, 3AcDON 78±4.8%, 15 AcDON 74±2.2% and NIV 81±3.8%, respectively. Detection limit for analyzed toxins was 0.01 mg/kg.

Results and discussion

Flour used in bread production is usually contaminated with mycoflora coming from grain, microorganisms constituting contamination of silos and milling equipment, as well as moulds coming from warehouses in which it is stored. The most dangerous microorganisms causing bread decay are spore-forming bacteria and toxin-producing moulds. Microbiological analyses of cereals in Poland showed that among the most frequently found mould microflora the dominant genera are *Aspergillus*, *Penicillium* and *Fusarium*, while every third isolated strain was toxin-forming [Packa et al., 2008; Tothill et al., 1992; Wiśniewska et al., 2004]. During bread baking only vegetative forms of microorganisms are destroyed, while both bacterial spores and mould spores remain in bread [Nowicki et al., 1988]. Mycotoxins as secondary metabolites are released to the medium and additionally they are thermostable compounds, which enhances the importance of the hazard connected with their occurrence. Thus baking does not guarantee microbiological stability or purity of the product [Osborne et al., 2007].

In this study contents of mycoflora and selected mycotoxins were analyzed in two spring wheat cultivars: Torka and Griwa. Grain samples were characterized by varied levels of mycoflora, in which the dominant species were fungi from genus *Fusarium*. Toxin-forming strains, producing mainly trichothecenes B, were identified as *F. culmorum* and *F. graminearum*. Samples of wheat cv. Torka were characterized by mean ERG concentration of 5.98 mg/kg. In case of cv. Griwa ERG concentration was 10.6 mg/kg (Table 1). The concentration of ERG in experimental grain was 4 times higher in case of wheat cv. Tork and 10 times higher in wheat cv. Griwa in relation to control samples. This shows an increased susceptibility of cv. Griwa to infection with microscopic fungi. All tested samples of inoculated grain exceeded ERG content proposed by European researchers as safe for consumers, i.e. 3 mg/kg [Schwadorf & Müller, 1989]. In the analyzed samples concentrations were determined for trichothecenes B, i.e. deoxynivalenol (DON), 3 – acetyldeoxynivalenol (3 –AcDON) and nivalenol (NIW). Mean total concentration of these toxins for wheat cv. Torka was 14.19 ug/kg, while for Griwy it was 18.63 ug/kg. Contents of DON in analyzed samples did not exceed the EU regulated concentration of 1250 ug/kg for unprocessed wheat grain (LIT).

Analyzed grain was milled under laboratory conditions, as a result of which flour type 750 and bran were produced (Table 1). Contents of both ERG and toxins in comparison to their contents in the parental material increased on average two times in bran, while in flour the content decreased by 90%. Flour produced in the course of milling was subjected to technological tests (Table 2). Statistically significant differences were observed between values of analyzed parameters between control and experimental samples. Acidity of flour from experimental grain in case of both cultivars was by approx. 50% higher, which was detectable during organoleptic examination. The amount and quality of gluten, which affects the formation of the porous structure in bread, was lower in flour from experimental grain. The next stage of the study included organoleptic examination and point score evaluation of bread following Standard PN – 92/A-74105 (Table 3). Acidity of bread was two times higher in bread from flour coming from experimental grain of wheat cv. Torka, while it was three times higher in cv. Griwa. The volume of bread baked from experimental samples was lower that the volume of control bread, in case of wheat cv. Torka by 25%, while for cv. Griwa by 33%. A similar dependence was observed by Olsson et al. [Olsson et al., 2002] in case of ochratoxin A. The content of this mycotoxin exceeded two-fold the admissible content of this toxin, i.e. 5 ng/kg, which had a significant effect on the quality of bread deteriorating their classification. The addition of mycotoxins to flour also deteriorated organoleptic properties of baked bread [Abbas et al., 1985].

Based on the conducted analyses considerable deterioration of bread quality was observed in case of samples characterized by the highest contents of mycotoxins and ERG. Thus it may be stated that an elevated content of mycoflora has a significant effect on the quality of cereal milling products and bread quality.

Table 1

ERG content (mg/kg) for two wheat cv. Torka and Griwa (experimental grain and control) and their milling products.

Cultivar	Type of sample	Grain		Bran		Flour	
		ERG (mg/kg)	Total toxin content (ug/kg)	ERG (mg/kg)	Total toxin content (ug/kg)	ERG (mg/kg)	Total toxin content (ug/kg)
Torka	Control	1.41	0.00	4.01	0.00	0.06	0.00
	Experimental	5.98	14.19	11.86	24.62	0.48	0.20
Griwa	Control	0.99	0.00	8.37	0.00	0.01	0.00
	Experimental	10.36	18.63	28.49	53.89	1.01	3.40

Table 2

Results of technological tests of wheat flour produced from cv. Torka and Griwa

Assay	Torka		Griwa	
	Control	Experimental	Control	Experimental
Total acidity [degree of acidity]	3.72	4.28	3.51	4.62
Moisture content [%]	14.62	12.82	14.11	12.10
Wet gluten content [g]	32.3	27.05	31.47	26.38
Gluten quality - elasticity	Very elastic	Low elasticity	Elastic	Low elasticity

Table 3

Results of quality testing of bread produced from wheat flour from wheat cv. Torka and Griwa.

Assay	Torka		Griwa	
	Control bread	Bread from experimental grain	Control bread	Bread from experimental grain
Bread acidity [degree of acidity]	1.35	3.18	1.54	4.87
Volume [cm^3/100g]	320	245	335	225
Quality class of bread	I	II	I	III
Yield of dough [%]	160.0	147.2	158.7	137.33
Yield of bread [%]	138.7	121.4	135.4	120.7

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8

ERGOSTEROL CONTENT IN CEREALS AND CEREAL PRODUCTS

Introduction

Chemical methods of analyses for specific markers of microscopic fungi have found a wide range of applications in recent years at various stages of quality control processes in foodstuffs of plant origin [Perkowski et al., 2008]. Apart from chitin and adenosine triphosphate, ergosterol (ERG) is one of the most important and most frequently used indicators of contents of microscopic fungi. ERG is the primary sterol found in the cell membranes of moulds, while it is absent in cells of the plant matrix [Müller & Lehn, 1998]. The assay method of ERG as an indicator of the presence of mycoflora in plant material was applied for the first time by Seitz et al. [1977]. This method has found extensive applications for the determination of fungal biomass in cereal grain [Perkowski et al., 2008, Perkowski & Miedaner, 1994], milling products [Perkowski et al., 2008], vegetables [Davis & Lamar, 1992; Kadakal et al., 2005], food products of plant origin [Dayal et al., 1991], soil [Ruzicka et al., 2000], feeds [Maupetit et al., 1993; Schnürer & Jonsson, 1992], building materials [Lau et al., 2006], dusts and aerosols [Axelsson et al., 1995; Saraf et al., 1997]. The occurrence of a characteristic absorption spectrum in the ultraviolet range makes it possible to distinguish it from other sterols found in plants [Müller & Lehn, 1998], while the obtained low detection limit of 0.01 mg/kg facilitates monitoring of even slight amounts of mycoflora in the analyzed material. Analysis of ERG content reflects the level of not only live fungal biomass, but also dead mycoflora found in the analyzed sample. Content of ERG is significantly correlated both with the content of mycotoxins and colony forming units (CFU), as it is indicated by analyses conducted to date [Miedaner & Perkowski 1995; Perkowski & Miedaner, 1994]. Thus on the basis of the concentration of this metabolite in the analyzed material the level of contamination with microscopic fungi may be determined. The presence of microscopic fungi is inseparably connected with the occurrence of secondary metabolites produced by these fungi, referred to as mycotoxins. In literature on the subject their toxic action has been proven in relation both to humans and animals. Mycotoxins enter the human organism first of all through the alimentary tract and when inhaled by people staying in mouldy rooms or warehouses where mould-contaminated material is stored (grain silos, mills, etc.). The alimentary portal of infection may be primary and secondary in character. The former is connected with consumption of mouldy food, while the latter concerns animal origin food contaminated with mycotoxins as a result of feeding animals with mycoflora-contaminated feed. The primary path seems rather non-significant, since people do not consciously consume food with visible signs of mouldiness. However, as it was shown in studies it is a very serious source of mycotoxins in the human organism. This results first of

all from the inappropriate treatment of mouldy food. In case of cereal products the level of mycoflora in the raw material has a significant effect on their quality. Microscopic fungi infesting kernels are responsible e.g. for deterioration of grain technological value, since by penetrating from the outer layer towards the centre of kernels they interact chemically with grain components, particularly those building the floury endosperm. Among others, enzymes (lipases, proteases, carboxylases) are responsible for this process. As a result of their action the structure may be partly or completely destroyed or new chemical substances may be formed, frequently providing a characteristic aroma or taste to the product [Gessner & Schmitt, 1996]. Thus it may be expected that during milling cereal products containing hulled kernel envelopes (e.g. bran, wholemeal flour) will be characterized by higher contents of fungal biomass than those produced from the inner kernel layers (e.g.: flour type 350, cake flour).

This study presents mean contents and range for ERG in grain samples of five staple cereals, i.e. wheat, triticale, rye, oat and barley. Contents of this metabolite were also analyzed in milling products of wheat, durum wheat, barley, rye, oat, maize, rice and buckwheat. The aim of the conducted investigations was to determine the level of mycoflora contamination of cereal grain for human consumption as well as cereal processing products available in retail in north-western Poland.

Material and methods

The analyzed material comprised 155 samples of 1000 g each of the following cereals: wheat (n = 35), triticale (n = 30), rye (n = 30), barley (n = 30) and oat (n = 30). Moreover, analyses were conducted on 261 samples of 1000g each of processing products of wheat, durum wheat, barley, rye, oat, maize, rice and buckwheat. Analyses were made on products produced as a result of diverse technological processes, i.e. flours of different extract grade, groats, flakes, bran and pasta. All samples came from north-western Poland. Grain samples were collected from grain elevators directly after harvest, while milling products came from retail outlets. The level of mycoflora in the above mentioned samples was determined by the chemical ERG analysis.

Analysis of ergosterol

For analyses samples of approx. 10 g were collected, from which after comminution in a laboratory mill (WŻ-1) samples of 0.1 g were collected for further analyses. Samples were placed in sealed test tubes of 17 cm^3, in which ergosterol extraction was run with simultaneous saponication. These processes occurred under the influence of microwave radiation. For this purpose methanol (2 cm^3) and 2 mol aqueous sodium hydroxide solution (0.5 cm^3) were added. Tightly sealed culture test tubes for safety reasons were placed in plastic bottles, which in turn were placed in a microwave oven (Whirlpool model AVM 401/1/WH, 2450 MHZ, 900W). Samples were exposed to microwave radiation of 350W in two sessions of 20 s each. After cooling (approx. 15 min) samples were neutralized using 1 mol aqueous hydrochloric acid solution. Next after an addition of 2 cm^3 methanol ergosterol was extracted using pentane (3 x 4 cm^3). Pentane extracts were collected and combined in a vial of 8 cm^3 and evaporated to dryness in a stream of nitrogen. Prior to analysis samples were dissolved in 1 cm^3 methanol. Analyses were conducted using a high performance

liquid chromatograph (Waters Alliance 2695) with an absorptiometric diode detector (Waters 2996 Photodiode Array Detector). Chromatographic separation was run in a Nova-Pak C18 column (150 mm x 3.9 mm), with a mixture of methanol and acetonitryle at a ratio of 90:10 (v/v) used as the elution phase. Ergosterol concentration was measured using an internal standard at a wave length λ= 282 nm. Compound identification was performed by comparing retention time of the analyzed peak with the retention time of the original standard and by adding a specific amount of the standard to the analyzed sample and repeated analysis.

Results and discussion

The postulate of obtaining possibly the lowest ERG concentration in milling products of the primary raw material for the production of food and feed, i.e. cereal grain, is fully justified. ERG content determined and presented in Table 1 for cereal grain for human consumption ranged from slight amounts (0.55 mg/kg) to several dozen milligram per kilogram (20.37 mg/kg). The lowest ERG content was found in wheat grain, followed by grain of triticale, rye, barley and oat. Concentrations of this metabolite in grain were also analyzed by Schnürer and Jonsson [1992], who found the range from 0.32 to 4.40 mg/kg for wheat, for triticale mean ERG concentration was 5.20 mg/kg, while for rye it was 5.10 mg/kg. In case of studies conducted by these authors, wheat was also characterized by the lowest mean ERG concentration, while the other cereals contained more of this metabolite in the following order: triticale, rye, barley and oat [Buśko & Wiśniewska, 2005]. Montgomery et al. found the highest ERG concentration in wheat grain (3.65 mg/kg) than in barley grain (2.14 mg/kg) [Miller & Young, 1983]. Similar results were reported by Zhang et al. Wheat grain analyzed in their study contained 8.63 mg/kg ERG, while barley grain – 4.14 mg/kg. Based on the conducted studies it may be stated that it is justified to classify microbiological quality of grain based on the concentration of ergosterol. In studies presented by Müller and Schwadorf [1990] and Maupetit et al. [1993] it was proposed to adopt the range of ergosterol concentration from 1 to 9 mg/kg as the range of concentration advisable for healthy grain. In turn, Schnürer and Jansson in 1992 based on their extensive analyses adopted the value of 3 mg/kg as the threshold value for grain for consumption purposes.

Analyzed milling products were characterized by low ERG concentrations (Table 2). Only the mean content of the analyzed metabolite in bran samples exceeded the concentration of 3 mg/kg ERG by over ten-fold. In all analyzed samples mean ERG content remained at a relatively low level ranging from 0.10 to 2.44 $mg \cdot kg^{-1}$. Among analyzed flours the highest ERG content was found for dark rye flour and wholemeal flour, which contained 2.44 mg/kg and 2.12 mg/kg ERG, respectively. The lowest concentration of this metabolite was found for rice flour, amounting to 0.10 mg/kg. Among groats the highest content of the analyzed microflora indicator was found for buckwheat groats, while the lowest for semolina. When comparing results recorded in this study with literature data we may observe that e.g. for wheat flour Müller and Schwardorf in 1990 recorded concentrations ranging from 4.0 $mg \cdot kg^{-1}$ to 35.8 $mg \cdot kg^{-1}$, Müller et al. 11.7 $mg \cdot kg^{-1}$, while Stuper and Perkowski [2008] 0.34 mg/kg.

It was found that the method, presented in this study and used to determine ergosterol content in plant material as an indicator of fungal biomass, makes it possible to analyze

different types of samples, including cereal materials and processing products. Based on the conducted studies it may be stated that contamination of grain for human consumption as well as cereal products in north-western Poland is low in comparison to literature data coming from other regions of Europe. The method applied in this study facilitates rapid assessment of contamination with microscopic fungi of tested material. The slight amounts of required reagents make this method relatively inexpensive and the wide range of concentrations facilitates the determination of contents of fungal biomass found even at a slight concentration. The method presented above may be commonly used in laboratories involved in analyses of cereals and cereal products.

Table 1

ERG contents [mg/kg] in samples of naturally infested cereals

Type of cereal	Number of samples	ERG concentration [mg/kg]	
		range	mean
Wheat	35	0.55 – 7.18	3.21
Triticale	30	1.05 – 11.24	5.70
Rye	30	1.84 – 14.27	7.29
Barley	30	2.16 - 17.11	9.38
Oat	30	4.97 – 20.37	11.92

Table 2

ERG concentration [mg/kg] in cereal processing products

Type of product	No.	ERG [mg/kg]
Corn flour	20	0.45
Wholemeal flour	30	2.12
Rice flour	7	0.10
Rye flour: dark	10	2.44
Barley flour	10	0.40
Wheat flour	60	0.42
Semolina	12	0.12
Corn grits	15	0.27
Buckwheat groats	12	0.97
Pasta from durum wheat	15	0.20
Pasta from bread wheat	15	0.15
Oat flakes	30	1.32
Bran	25	31.72

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CHAPTER 3

CREATION AND DETERMINATION OF FOOD QUALITY

1

CONTENT OF SOME SELECTED BIOLOGICALLY ACTIVE COMPOUNDS AND THEIR INFLUENCE ON ANTIOXIDANT PROPERTIES OF TOMATO PRODUCTS

Introduction

At present, there is a tension that plant-origin products to have large amounts of antioxidants that effectively protect against the civilization diseases. Tomato is abundant in compounds with strong antioxidant capacity and the epidemiological survey univocally indicates that it diminishes the risk of having some tumor types such as prostatic carcinoma [Chang et al., 2006; Giovannucci et al., 1995]. Among compounds present at tomato, lycopene is characterized by the greatest biological activity, although it is also an abundant source of other carotenoids such as α-carotene or β-carotene, as well as L-ascorbic acid, flavonoids, and phenolic acids [Abushita et al., 2000; Chang et al., 2006; George et al., 2004]. Tomatoes are the most frequently consumed as raw fruits, but also in processed forms like juice, paste, or sauce (e.g. ketchup), that are the most important lycopene source in a diet. In Poland, tomato juice is the most often consumed vegetable juice.

The carotenoids contents in tomato depend, among others, on such factors as: climatic conditions, nutrition, harvest date (ripeness), and storage conditions [George et al., 2004], whereas lycopene contents are influenced on by soil fertilization with potassium and phosphorus [Fish et al., 2002]. Thermal processing has not any significant effects on lycopene level, instead it makes the lycopene can be more readily absorbed in an organism as compared to raw products, although affects the considerable decrease of L-ascorbic acid content, that is the strongest water-soluble antioxidant [Perez-Conesa et al., 2009; Saint-Cricq de Gaulejac et al., 1999; Toor & Savage, 2006] ; the polyphenols concentrations in a final product depends on a technological process applied [Aisling-Aherne & O'Brien, 2002]. Changes in these compounds contents in a final product have the influence on its antioxidant capacity.

The study aimed at evaluating the concentration of biologically active substances in tomato products as well as determining their impact on the products' antioxidant capacity.

Materials and Methods

Material for study consisted of three tomato juice types and three tomato pastes available on Warsaw markets. In order to indicate the difference among studied juices, one juice was a brand made of fresh tomatoes (S1), while two others were cheaper and produced from a puree (S2 and S3).

Determination of ascorbic acid content by means of xylene method using 2,6-dichlorophenoloindophenol was performed according to norms. The lycopene was analyzed applying spectrophotometry [Chang et al., 2006; Fish et al., 2002] based on lycopene extraction with BHT in acetone: ethanol: hexane mixture (1:1:2) from studied sample and measuring the absorbance at 503 nm wavelength of hexane solution. The lycopene content in examined juice was calculated with a help of extinction coefficient (E%) for lycopene in hexane as 3120 at wavelength used. Because the color of tomato products is strictly associated with the lycopene contents, the color was measured using MINOLTA CR-200 device adjusted to white standard. The amount of total polyphenols (TPC) in extracts was determined with the Folin-Ciocalteau's reagent [Naczk & Shahidi, 1989]. Absorption at 700 nm was measured (Shimadzu UV-160A). The content of total polyphenols was expressed as gallic acid (GA) (Sigma Chemicals Co.) equivalents in g of GAE /100 g d.m.

Methanol extracts (15 g products: 100 cm^3 methanol) were prepared to determine the antioxidant capacity by means of cation-radicals $ABTS^{\bullet+}$, stable radicals $DPPH^{\bullet}$, and ability to chelate the iron (II) ions by hydrophilic substances. Extracts of hydrophobic substances to determine of the ability to deactivate the stable $DPPH^{\bullet}$ radicals were prepared analogously.

The antioxidant capacity of extracts was also determined by measuring their ability to inactivate the stable synthetic $DPPH^{\bullet}$ radicals [Saint-Cricq de Gaulejac et al., 1999]. Extract with antiradical compounds was added into the $DPPH^{\bullet}$ solution that showed absorbance at 517 nm in its radical form. The antiradical activity was calculated on a base of the difference between $DPPH^{\bullet}$ solution absorbance values before and after antioxidants addition, and expressed in %.

The antiradical activity of extracts was determined against cation-radicals $ABTS^{\bullet+}$ (produced in a reaction of 2,2'-azino,bis-3-ethylbenzothiazoline-6-sulfonic acid with potassium hypersulfate) [Re et al., 1999]. The absorbance measure was made at 734 nm, and the results were expressed as Trolox equivalents (µmol TEAC/100g or 100 d.m.).

The ability to chelate the iron (II) ions was assessed by means of spectrophotometry [Lai et al., 2001]. Ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt, Sigma Chemicals Co.), when added during the determination, forms a color complex with iron (II) ions. Its absorbance was measured at 562 nm (Shimadzu UV-160A). The ability of extracts to bind iron (II) ions was expressed in %.

Achieved results were subjected to statistical processing applying Statgraphics Plus 2.1 software; the difference significance between mean values was verified using LSD test ($p \leq 0.05$), as well as two-factorial analysis was carried out resulting in correlation coefficients calculation.

Results and Discussion

In order to determine the lycopene content, the spectrophotometric method was applied, for which a high accuracy comparative with HPLC technique, was found [Rao et al., 1998]. Analysis of determined lycopene contents (Table 1) revealed their significant differences in analyzed tomato products. Among juices, S1 made of fresh fruits contained the largest amounts of lycopene (8.92 mg/ 100g), while S2 produced from concentrated tomato puree – the smallest (7.26 mg%). Achieved results are within the range for juices, i.e. 5.18- 9.74 mg% [Hamułka & Wawrzyniak, 2004]. Other research confirmed its higher content – 10.77 mg% [Tonucci et al., 1995]. Among examined pastes, K2 contained the most lycopene (45.6 mg%), whereas K1 the least (30.41 mg%). Here achieved results are within the lycopene range (20.94- 49.33 mg/100 g) set by Hamułka and Wawrzyniak [2004].

Table 3

Content of lycopene in tomato products

Product	Lycopene	
	mg/100 g	mg/100g d.m.
S1	$8.92^{a} \pm 0.02$	$118.2^{a} \pm 0.2$
S2	$7.26^{b} \pm 0.02$	$103.9^{b} \pm 0.2$
S3	$7.77^{c} \pm 0.02$	$115.5^{c} \pm 0.3$
K1	$43.77^{d} \pm 0.02$	$131.1^{d} \pm 0.1$
K2	$45.60^{e} \pm 0.10$	$138.8^{e} \pm 0.3$
K3	$30.41^{f} \pm 0.04$	$84.3^{f} \pm 0.1$

Explanatory notes:
± standard deviation; a-e -the same letters in column show no significant differences between means ($p \leq 0.05$)

Comparison of that component levels recalculated onto dry matter revealed that pastes K1 and K2 contained much more lycopene than examined juices. Its concentration was much higher in pastes than juices due to a great resistance of the compound towards technological process parameters [Abishita et al., 2000]. Moreover, lycopene is released from the cells during their damage and thermal processing, which makes easier to its extraction and inactivation of oxidizing enzymes such as peroxidase able to oxidize carotenoids when released from the cells [Perez-Conesa et al., 2009]. The influence of these factors, i.e. breaking size and thermal processing, made that lycopene content was higher in tomato products than in raw tomato fruits [Shi et al., 2008].

According to the literature references [Arias et al., 2000], lycopene content at tomato is strongly correlated with +a* parameter describing the share of red color in Hunter's system. These authors indicated the possibility to apply that simple method of tomato red color measure to determine the antioxidant content and to predict the antioxidant capacity of tomato due to a strong correlation between both parameters. Therefore, the assessment of examined products color was made (Table 2) to verify if the dependence could be also applied for tomato products.

Table 4

The L^* a^* color factors of tomato juices

Product	L	a^*
S1	$30.8^{a} \pm 0.6$	$23.6^{a} \pm 0.2$
S2	$30.6^{a} \pm 0.1$	$23.6^{a} \pm 0.4$
S3	$30.8^{a} \pm 0.4$	$16.1^{b} \pm 0.5$
K1	$30.0^{ac} \pm 0.2$	$39.8^{c} \pm 0.9$
K2	$32.3^{b} \pm 0.8$	$38.6^{cd} \pm 1.7$
K3	$29.5^{c} \pm 0.1$	$37.3^{d} \pm 0.4$

Explanatory notes:
± standard deviation; a-d -the same letters in column show no significant differences between means ($p \leq 0.05$)

Brightness of studied juices and pastes was similar ranging 29.5-32.3. These were lower values than those achieved for tomato by Wold et al. [2004]. Among juices, S1 and S2 were characterized by the most intensive red color (statistically insignificant differences), and the parameter value was 23.6; the lowest share of red color was represented by S3 sample (16.1). Pastes were characterized by much higher +a* value; however the differences were not significant. Only for K1 and K3, statistically significant differences amounting to 39.8 and 37.3 were found. These products had more intensive red color than juices, probably due to their higher concentration level and higher lycopene contents. Thus, the dependence between parameters was searched for on a base of achieved +a* value and lycopene content. Significant dependence (R^2=0.88) between trichromatic coordinate corresponding to red color vs. lycopene content in a product was observed for analyzed materials. An association between color and lycopene concentration at tomato was also recorded by Arias et al. [2000], who found that the linear correlation between those parameters was R^2=0.82, while exponential correlation between +a* parameter and lycopene content was stronger (R^2=0.96). Hence, it can be concluded that tomato's color does not considerably change during any technological processing, although it is known that due to dehydration during processing, a hydroxymethylfurfural (HMF) is formed from sugars, as well as lycopene losses can be observed. However, HMF amounts are very low or it is absent, thus its influence on color changes at tomato products can be neglected [Porreta & Sandei, 1991]. Instead, great changes of lycopene contents were observed during such processes as frying accompanied by a^* value decrease [Sahlin et al., 2004].

Table 5

Content of total polyphenols and ascorbic acid in tomato products

Product	Total polyphenols		Ascorbic acid	
	mg/100 g	mg/100 g d.m.	mg/100 g	mg/100 g d.m.
S1	$68.08^{a} \pm 2.50$	$901.59^{a} \pm 33.21$	$17.57^{a} \pm 0.28$	$228.39^{a} \pm 3.70$
S2	$74.19^{a} \pm 2.15$	$1061.31^{b} \pm 30.75$	$5.30^{b} \pm 0.06$	$75.83^{b} \pm 0.87$
S3	$53.24^{b} \pm 0.54$	$790.70^{c} \pm 7.96$	$3.40^{c} \pm 0.36$	$50.54^{c} \pm 5.38$
K1	$193.69^{c} \pm 11.30$	$580.31^{d} \pm 33.86$	$15.78^{d} \pm 0.67$	$47.29^{c} \pm 2.02$
K2	$163.82^{d} \pm 4.17$	$498.78^{e} \pm 12.71$	$12.80^{e} \pm 0.12$	$38.98^{d} \pm 0.37$
K3	$175.36^{e} \pm 1.79$	$486.14^{e} \pm 4.96$	$10.22^{f} \pm 0.12$	$28.33^{e} \pm 0.53$

Explanatory notes:
± standard deviation; a-f -the same letters in column show no significant differences between means ($p \leq 0.05$)

From 53.24 do 193,69 mg/100 g polyphenols depending on the product, were determined in examined tomato juices and pastes. Juices S1 and S2 contained the largest amounts of polyphenols (statistically insignificant differences), while S3 – the smallest (55.46 mg/100 g). According to literature data, these compounds contents in juices should range within 30.12- 52.26 mg/100 g [Podsędek et al., 2003]. Here achieved results are higher, which may resulted from the application of more concentrated extracting agent: in cited work, authors used 50% methanol, while here an absolute methanol was applied to extract polyphenols. It is well know that the results of polyphenols determination are influenced by the extraction conditions, i.e. solvent, extraction duration, and material to solution ratio. Studies performed by Chavan et al. [Chavan et al., 2001] revealed that the increase of methanol concentration from 70% to 100% improved the polyphenols extraction efficiency. Thus, it could be supposed that 50% methanol is less effective in that process. However, it cannot be excluded that the differences were due to different amounts of these substances in raw materials, because their levels in fresh material may vary from 30 to 80 mg% [Proteggente et al., 2002; Wu et al., 2004]. Analysis of results achieved for studied pastes revealed that K3 was the most rich in polyphenols (193.69 mg/100 g), while K2 – the least abundant (163.82 mg/100 g). As similar as in the case of juices, these results are slightly higher than 116.74–155.44 mg/100 g found in earlier research [Podsędek et al., 2003], which may resulted from distinct extraction conditions. When comparing the polyphenols contents recalculated onto dry matter, it becomes apparent that it was almost twice as low in pastes as in juices. Aisling-Aherne et al. [2002] reported that phenolics losses may reach up even 50% at vegetables subjected to such processes as pasteurization, sterilization, or freezing.

Concentrations of L-ascorbic acid in examined tomato juice and paste samples varied oscillating within the wide range from 3.40 to 17.57 mg/100 g. Juice S1 contained the largest quantities of vitamin C (17.54 mg/100 g) produced by the raw material itself, and it was similar value to that in raw tomato (15–21 mg/100 g) [Abushita et al., 2000]. The lowest L-ascorbic acid level was found in S3 (3.54 mg/100 g), that was made of a concentrated tomato puree. According to literature, content of vitamin C in juices with no its addition may vary within the range of 7.65–23.6 mg/100 ml and the differences found between different juices may result, besides technological process conditions, also from the package type [Sanchez--Moreno et al., 2006]. Analysis of studied pastes revealed that K3 contained the highest amounts of L-ascorbic acid (15.78 mg/ 100 g), while K1 – the lowest (10.22 mg /100 g), and the values ranged from 14.47 to 21.56 mg/100 g [Podsędek et al., 2003]. Great differences in the compound contents in pastes may resulted from its different levels in the raw material, but mainly from processing temperature and storage conditions. Depending on the temperature applied during the technological process, L-ascorbic acid content losses can decrease, e.g. during hot breaking and pasteurization, even up to 90% [Perez-Conesa et al., 2009].

Among studied juices, the strongest ability to deactivate the DPPH$^{\bullet}$ radicals was shown by methanol extract components of S1, the activity of which was very similar to values achieved for the pastes, i.e. 96.8%. For S3, the lowest antioxidant capacity was recorded (58%). Referring to pastes, the strongest antiradical activity was manifested by K3 sample (99.5%), while K1 and K2 were characterized by slightly lower values (about 98.5%). Zhong and Yu [2006] reported that the activity of substances extracted from acetone plus water mixture (1:1) from raw tomatoes, against DPPH$^{\bullet}$ radicals, ranged within 15–60%. Such large differences in achieved results for examined products and raw fruits may resulted from different solvents used for extraction, but also the fact that products had been

processed. Methanol and its water mixtures are most often used to extract hydrophilic substances, whereas other solvents, e.g. acetone, are characterized by lower efficiency [Kalt et al., 1999]. Temperature is another factor that affects the achieved results; thermal processing contributes to the decrease of these compounds concentration, and on the other hand, it makes that cell membranes become more permeable. In consequence, it results in higher extraction efficiency for processed than raw tomatoes [Perez-Conesa et al., 2009]. Research performed by Chang et al. [2006] confirmed that observation, because they reported the activity against DPPH$^{\bullet}$ radicals of substances extracted with methanol from raw then dried tomatoes at the level of about 97–99%.

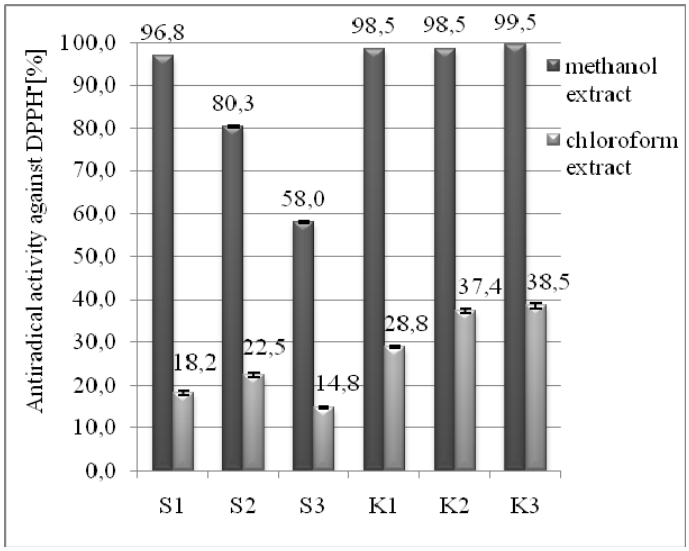

Fig. 7. Activity of methanol and chloroform extract of tomato products against DPPH$^{\bullet}$ radicals

Analysis of analogous results for the substances in chloroform extracts revealed that they were characterized by much lower ability to inactivate stable DPPH$^{\bullet}$ radicals than those contained in methanol extracts. Similar results were also achieved by Sanchez-Moreno et al. [Sanchez-Moreno et al., 2006], who a high activity against these radicals in hydrophilic extracts attributed to vitamin C. Among examined pastes, compound extracted from K3 showed the strongest ability (38.5%), while those extracted from K1 – the weakest (28.8%). Referring to juices, S2 showed the most intensive antiradical activity (22.5%), whereas S3 – the least (14.8%), which was associated with higher content of lycopene that shows the strongest activity against DPPH$^{\bullet}$ radicals among all carotenoids [Jiménez-Escrig et al., 2000]. A positive correlation r=0.82 ($p \geq 0.95$) between its concentration and antiradical activity can confirm it.

Comparison of activity against $ABTS^{\bullet+}$ radicals results expressed in Trolox equivalents (μmol TEAC/100 g) revealed that it was about twice as high in juices as in pastes. Achieved results for juices are higher than the range for hydrophilic extracts made of such products (98-179 μmol TEAC/100 g) [Podsędek et al., 2003] and the range of 2549–2579 μmol TEAC/100 g d. m. [Toor & Savage, 2006] for raw tomatoes. It may result from the type of the extraction agent applied. The former study involved 50% methanol that is

characterized by lower polyphenols extraction efficiency, the latter used 70% acetone, in which ascorbic acid is unstable. When comparing the activity results recalculated onto dry matter, it is apparent that the antiradical activity was much lower in pastes rather than juices, which resulted from a lower contents of hydrophilic components, i.e. polyphenols and L-ascorbic acid, the presence of which determines the behavior against $ABTS^{\bullet+}$ radicals. A positive correlation between polyphenols contents vs. the activity (r= 0.95) is the confirmation. In the case of L-ascorbic acid, no such dependence was recorded.

Table 6

Activity of extracts of tomato products against $ABTS^{\bullet+}$radicals

Product	Antiradical activity µmol TEAC/100g	Antiradical activity µmol TEAC/100g dm
S1	$444.9^{a} \pm 5.8$	$5892^{a} \pm 76$
S2	$329.2^{b} \pm 8.0$	$4709^{b} \pm 114$
S3	$217.1^{c} \pm 8.5$	$3224^{c} \pm 126$
K1	$728.7^{d} \pm 1.8$	$2183^{d} \pm 35$
K2	$742.2^{d} \pm 12.2$	$2260^{d} \pm 37$
K3	$810.1^{e} \pm 7.4$	$2245^{d} \pm 20$

Explanatory notes:
± standard deviation; a-e -the same letters in column show no significant differences between means ($p \leq 0.05$)

Analysis of results upon the ability to chelate iron (II) ions (Fig. 2), it is obvious that both juices and pastes were characterized by similar features. Among juices, S2 showed the strongest chelating ability (98.9%), while S1 the weakest (96.9%). Among extracts from tomato pastes, the strongest chelating ability was manifested by K1 (98.9%), whereas the weakest – by K3 (98.0%). Lower results were achieved when examined raw and dried tomatoes (77 and 93%, respectively) [Chang et al., 2006].

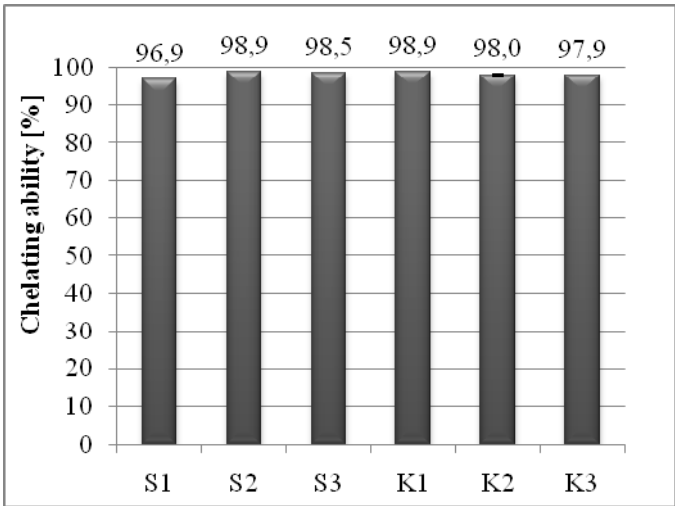

Fig. 8. Ability to chelate iron ions (II) [%]

It can be noted that processed tomato products had larger ability to chelate iron (II) ions than raw tomato fruits. Methanol was applied as extraction agent in presented study, hence the contents, availability of active substances in a product, and extraction time are the main factors that determine the iron (II) ions chelating ability.

Conclusions

All examined products were characterized by high contents of biologically active substances, i.e. lycopene and polyphenols. The lycopene concentration was higher in pastes due to its thermal stability and enhanced availability after technological processing, and it was positively correlated with the color parameter a*. The polyphenols quantity, recalculated onto dry matter indicated their considerable losses in pastes. The ascorbic acid content largely depended on the process applied, and it is the highest in juice made of raw tomatoes. Amount of active compounds is associated with product's antioxidant capacity. The strongest dependencies were found between $DPPH^{\bullet}$ radicals inactivation ability of the components contained in chloroform extracts and lycopene concentration, as well as ability to inactivate the $ABTS^{\bullet+}$ radicals by hydrophilic constituents vs. polyphenols contents.

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2

ANTIBACTERIAL ACTIVITY OF LYSOZYME DEPENDING ON DIMER CONTENT

Introduction

Lysozyme (E.C.3.2.1.17) is a relatively small enzyme (14400 Da), which catalyses hydrolysis of specific polysaccharides contained in bacterial cell walls. The primary, extremely rich source of lysozyme is hen egg white, in which it accounts for approx. 3.5% share of all contained proteins. As a component of the egg white in bird eggs this enzyme provides a natural protective barrier for egg contents against microorganisms. The protective action consists in the destruction of bacteria by disruption of β(1-4) bonds between N-acetylmuramic acid and N-acetylglucosamine, a copolymer of polysaccharide found in the cell wall of many bacteria. These natural properties of the enzyme have found an increasing number of applications e.g. in food industry, medicine and veterinary medicine.

Antibacterial activity of lysozyme pertains mainly to Gram-positive bacteria, since the exposed peptidoglycane, a substrate of lysozyme, is directly subjected to the action of this enzyme. Gram-negative bacteria are much more protected against its antibacterial action. The decomposition of cell walls is hindered due to the presence of additional components in their polysaccharide-peptide complex, such as polypeptides, lipoproteids and lipopolysaccharides (LPS), which form the protective layer. This layer constitutes for lysozyme a hardy permeable barrier and its destruction usually is sufficient to initiate the hydrolytic action of the enzyme. Antibacterial action of lysozyme, as well as its action against certain fungal species are used in food industry, medicine, veterinary medicine and pharmacology [Hughey and Johnson 1987; Kiczka, 1994; Kopeć and Trziszka, 1997].

It results from a review of literature that potential practical applications of lysozyme monomer are extensive and to a large extent utilized at present [Cegielska-Radziejewska et al., 2008; Ibrahim, 2003]. However, it is also known that lysozyme has many more valuable properties extending the range of its action, manifested primarily as a result of enzyme modification. At present in several research centres worldwide, including ours, intensive studies are being conducted on the generation of such a form of lysozyme, which would make it possible to fully utilize this enzyme.

When conducting studies on antibacterial properties of lysozyme, its action against Gram-negative bacteria has been repeatedly confirmed. This action is attributed mainly to the destruction of the lipopolysaccharide (LPS) layer, protecting the cell wall in these bacteria. Analyses showed that this effect may be obtained as a result of enzyme modification. Conducted investigations concerned first of all chemical and physico-chemical modifications of the enzyme. The former consist in the formation of bonds of lysozyme with substances cooperating with this enzyme in its bactericidal action [Ibrahim et al., 1994], while

the latter -mainly in thermal processes [Ibrahim et al. 1996, Leśnierowski et al., 2004]. It was shown in these studies that modifications of lysozyme, resulting in the production of dimer and higher oligomers, caused an increase in its biological bacteriostatic activity extending its antibacterial action, which creates an opportunity of a markedly wider utilization of the enzyme as a food preservative.

Due to conformational differences the enzyme loses partially its hydrolytic activity; however, it still retains antibacterial activity against Gram-positive bacteria and is effective against Gram-negative bacteria [Cegielska-Radziejewska et al., 2009; Leśnierowski et al., 2001, Leśnierowski et al., 2009]. It was found that antibacterial activity of lysozyme is independent of its catalytic functions, as it is evidenced by results of genetic studies on this enzyme [Ibrahim et al. 2001].

The aim of the study was to assess antibacterial properties of lysozyme preparations with different proportions of dimer in relation to selected bacterial strains.

Material and methods

Experimental material consisted of lysozyme monomer (by Belovo) and dimer (Lydium KLP by Nika). Anlayses were conducted in a model system. Lysozyme preparations were prepared containing 0, 20, 40, 60, 80 and 100% dimer and 100, 80, 60, 40, 20 and 0% monomer, respectively. Antibacterial activity was assessed using the disc-diffusion method. Bacteria were incubated for 24h on a broth medium. After that time bacterial suspension was prepared with a density of 0.5 McFarland using a Densimat apparatus (Biomérieux) and physiological saline NaCl Medium (Biomérieux). The amount of 100µl suspension was spread using a sterile spatula on the surface of Mueller – Hinton Agar medium (Oxoid). Plates were left for 15 minutes at 22°C. Blotting paper discs (Biomérieux) with a diameter of 6mm were placed on the surface of agar. Amounts of 10µl monomer and dimer mixtures were placed dropwise onto discs. Preincubation was run for 30 min at room temperature. Plates were incubated at 30°C and 35°C, depending on the type of bacteria, for the period of 24 h. After that time the size of growth inhibition zones was measured. In the experiments the following test strains were used: *Micrococcus luteus* by Calbiochem, *Pseudomonas fluorescens* PCM 2123/=NCTC 3756/ Institute of Immunology and Experimental Therapy in Wrocław.

Results were subjected to statistical analysis using *STATISTICA PL v.7.0* software.

Results

For model preparations of lysozyme prepared in a manner presented in Table 1 antibacterial activity was determined in relation to selected bacteria. In analyses Gram-positive and Gram-negative bacteria were used: *Micrococcus luteus* and *Pseudomonas fluorescens*. Bacteria from genus *Micrococcus* from the group of Gram-positive bacteria are sensitive to the action of lysozyme monomer and are used to assess hydrolytic activity of the enzyme. Bacteria *Pseudomonas fluorescens,* belonging to the category of psychrophilic bacteria, constitute the dominant microflora responsible for decay of many cold stored foodstuffs.

Table 1

Percentages of lysozyme monomer and dimer in analysed samples

Sample	Percentage of monomer [%]	Percentage of dimer [%]
1	100	0
2	80	20
3	60	40
4	40	60
5	20	80
6	0	100

It was shown that antibacterial activity of lysozyme in the analyzed system is dependent on the type of bacteria and the percentage of dimer in the mixture. The formation of growth inhibition zones was found in case of all analyzed lysozyme preparations for both tested bacteria. The dependence between the content of dimer in the tested preparation was shown both for *Micrococcus luteus* and *Pseudomonas fluorescens* bacteria. In case of Gram-positive *Micrococcus luteus* the most effective antibacterial action was observed for the preparations not containing lysozyme dimer (Fig.1). The smallest growth inhibition zone was found in case of a preparation containing 100% dimer.

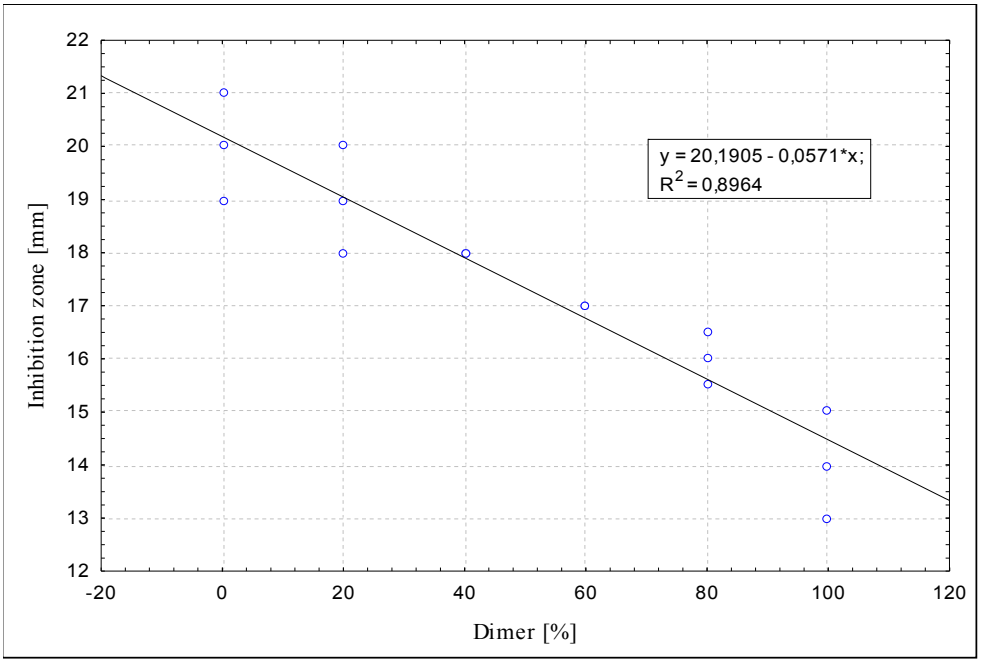

Fig. 1. A dependency of inhibition zone for *Micrococcus luteus* on the percentage of dimer in sample

An opposite dependence was shown for a strain *Pseudomonas fluorescens*, belonging to Gram-negative bacteria. The biggest growth inhibition zone of 20 mm was observed for a preparation containing 100% dimer. With a decreasing percentage of dimer content growth inhibition zones were observed to decrease to 9 mm (Fig. 2).

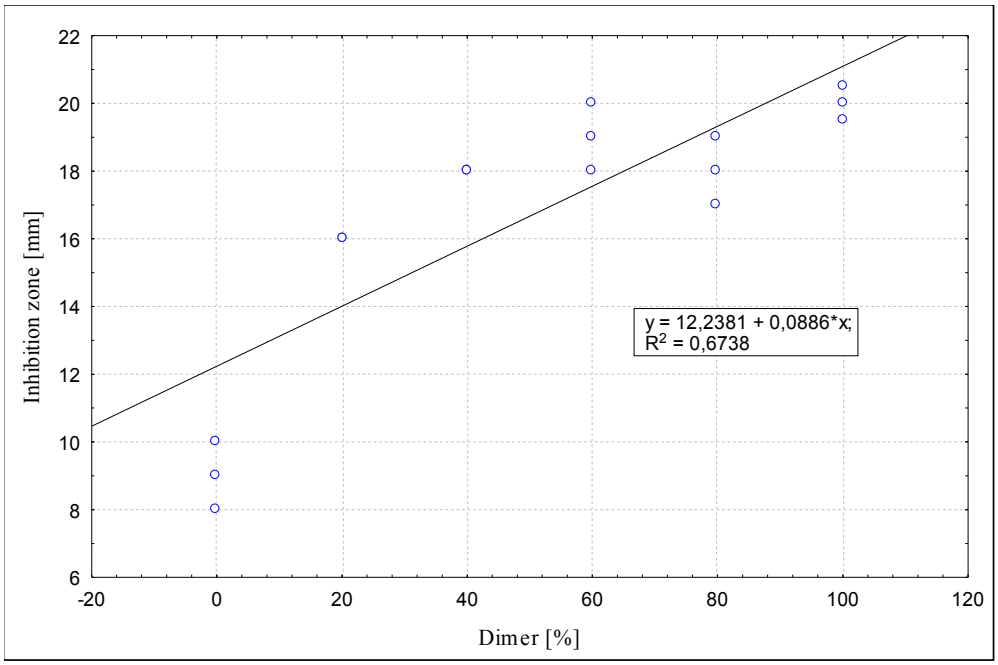

Fig. 2. A dependency of inhibition zone for *Pseudomonas fluorescens* on the percentage of dimer in sample

Based on the conducted regression analysis it may be stated that for both analyzed strains a statistically significant linear dependence was found between dimer content in the preparation and the size of growth inhibition zones. In case of Gram-positive bacteria the difference in the size of bacterial growth inhibition zones between samples with a 100% monomer content and 100% dimer content was smaller than in case of Gram-negative bacteria. It may be stated that dimer exhibits antibacterial activity also in relation to Gram--positive bacteria.

Earlier studies concerning model preparations of lysozyme indicate that an increase in the proportion of dimer in the preparation results in an increase in hydrophobic surface (ΔPH) and a simultaneous reduction of hydrolytic activity [Leśnierowski et al., 2006]. Despite a decrease in hydrolytic activity of lysozyme preparations with a reduced proportion of monomer, their antibacterial effectiveness in relation to Gram-negative bacteria *Pseudomonas fluorescens* increased. It may be stated that an increase in hydrophobicity of lysozyme preparations with an increased proportion of dimer results in an increase in antibacterial activity in relation to Gram-negative bacteria *Pseudomonas fluorescens*. At present studies are being conducted on the dependence between the size of hydrophobic surface of the lysozyme preparation and its activity in relation to Gram-negative bacteria.

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3

ANTIOXIDANT PROPERTIES OF GLOBULIN PREPARATIONS FROM THE SEEDS OF CHOSEN LEGUMINOUS SPECIES

Introduction

Oxidation processes, besides microbial changes, have an important influence on food quality during the whole production and storage cycle. Oxidation reactions of lipids, that are main and the least stable components, contribute to the decrease of nutritional value and worsening the sensory properties of foodstuff. In order to reduce the negative effects of these processes, the addition of antioxidants into the food is often applied. Discovering in toxicological assays the threats for human's health by synthetic inhibitors of oxidation processes (BHA, BHT) led to greater tendencies of their replacing with natural substances. Numerous studies proved that besides polyphenols, tocopherols, carotenoids, and ascorbic acid, peptides and proteins also show very good antioxidant features [Chen et al., 1998; Hunt et al., 1988].

Legume seeds are a potential source of protein antioxidants, namely due to their high contents. It is important that protein preparations made of legumes found a wide application as food additives because of their good functional properties. Most of published works refer to antioxidant capacity of proteins and hydrolyzates made of soybean seeds [Pena-Ramos and Xiong, 2003; Ulu, 2004]. Promising results of those studies resulted in the need to verify the efficiency of oxidation inhibition invoked by proteins isolated from other legume seeds such as bean, pea, or broad bean.

Materials and Methods

The protein preparations were achieved from seeds of pea [Poa, *Pisum sativum*), broad bean (Bartom, *Vicia faba*), white runner bean (Piękny Jaś tyczny*, Phaseolus multiflorus*), as well as white common bean (Prosna, *Phaseolus vulgaris*), brown common bean (Nida, *Phaseolus vulgaris*), and red common bean (Red Kidney, *Phaseolus vulgaris*). Seeds were de-fatted, crushed, and sieved (100 mesh). Preparations were produced by means of isoelectric protein precipitation from alkaline flour extracts (pH 9.2) and centrifuging the protein sediment that was subsequently dissolved in distilled water (pH 7.0) and lyophilized.

Protein preparations were denoted in the study by the following abbreviations:
BP – broad bean preparation,
PP– pea preparations,
WBP-PJT – white runner bean var. Piękny Jaś tyczny preparations,

WBP-P – white common bean var. Prosna preparations,
BBP – brown common bean preparations,
RBP – red common bean preparations.

The study presents the characteristics of protein preparations including protein contents determination [AOAC, 1990] and available thiol groups in a reaction with 2,2`-dithiobis (5-nitropyridin) (DTNP) by means of spectrophotometry at λ=386 nm wavelength [Martinaud et al., 1997; Soyer and Hultin, 2000], protein's surface hydrophobicity with 8-anilin-1-naphthalenesulfonic acid (ANSA) applying spectrofluorimetry at λ_{wz}=390 nm and λ_{em}=479 nm [Hayakawa and Nakai, 1985].

The SE-HPLC method [Musakhanian and Alli, 1987] was applied to determine the molecular weight of proteins in preparations. Protein separation was made on TSK G2000SW LKB column (0.75 × 60 cm), while their detection was done at 280 nm (UV SPD-6A detector, Shimadzu). Phosphate buffer (0.1 M; pH 7.0) and NaCl (0.5M) at 0.5 ml/min flow rate were used for elution. Aliquots of 20µl filtered samples (Supelco filter) were introduced onto the column. Following molecular weight standards (Pierce) were used to calibrate the column: cytochrome (12.5 kDa), chymotrypsinogen (25 kDa), egg albumin (45 kDa), bovine albumin (67 kDa), catalase (158 kDa), ferritin (240 kDa), and Blue Dextran (2000 kDa).

The antiradical activity of examined protein preparations (with an addition of 100 mg% soluble protein) was determined against hydroxyl radicals that were produced in a mixture containing Cu(II)/H_2O_2/sodium benzoate/DTET [Hunt et al., 1998). The antiradical activity against hydroxyl radicals was calculated (%) on a base of the fluorescence intensities (λ_{wz}=308 nm; λ_{em}= 410 nm) due to benzoate hydroxyl derivatives formed in the reaction.

Moreover, the antioxidant capacity of globulin preparations were tested on a base of their addition influence on inhibition of linolic acid emulsion oxidation (catalyzed by hemoglobin addition), then spectrophotometric measuring the produced peroxides at λ=480 nm [Kuo et al., 1999], and then recalculating achieved absorbance results onto the activity expressed in per cents.

Recorded results were subjected to statistical processing with a help of Statgraphics Plus 3.0 software and calculating linear correlation coefficients as well as statistical significance of differences (LSD test).

Results and Discussion

A general characteristics of studied preparations is presented in Table 1. Applied method for protein isolation at the lowest solubility point made possible to achieved high-protein preparations (70-87.9% d.m), that considerably varied between particular species of examined legumes. The lowest protein amount was found in preparations made of bean var. Prosna (70%), in other bean preparations, it amounted to about 76% d.m. Preparations achieved from pea and broad bean seeds were characterized by higher protein contents (84.6 and 87.9% d.m, respectively).

Table 1

The characteristic of protein preparations from broad bean, pea and bean

Preparates	Total protein content [% d.m.] N x 6,25	Soluble protein content [%]	Surface aromatic hydrophobicity [j.u. FI/% protein]	Available thiol groups content [μmole -SH/g protein]
BP	87.9 (±0.1)	36.6 (±0.3)	180.8 (±2.5)	11.5 (±0.2)
PP	84.6 (±0.3)	53.7 (±0.5)	229.5 (±7.6)	12.2 (±0.2)
WBP-PJT	76.0 (±0.5)	44.8 (±0.2)	363.5 (±8.2)	11.1 (±0.2)
WBP-P	70.1 (±0.5)	45.2 (±0.6)	142.1 (±2.2)	14.8 (±0.4)
BBP	76.6 (±0.6)	49.2 (±1.1)	151.9 (±2.2)	14.3 (±0.2)
RBP	76.9 (±0.3)	48.3 (±0.4)	132.0 (±2.7)	12.6 (±0.1)

The antioxidant capacity of protein preparations are associated, among others, with sulfur amino acid content, because thiol groups contained, take part in free radical scavenging [Tong et al., 2000]. Proteins of the preparations investigated showed the availability of thiol groups on the level of 11–15 SH μM/g of protein. The highest concentration of thiol groups (15 μM SH/g protein) was recorded in white bean var. Prosna. Availability of thiol groups in preparations is not consistent with sulfur amino acid contents in globulins of studied legumes, slightly larger amounts of which are present in globulin 11S, i.e. fraction dominating in broad bean and pea proteins, as compared to the main fraction of common bean (globulin 7S). It probably results from the fact that part of them forms disulfide bridges binding acidic with alkaline subunits within globulin 11S [Kulka and Grzesiuk, 1978]. The results obtained in the study concerning available thiol groups in globulin preparations of legume seeds investigated are lower than amounts of these groups in pea and bean albumins preparations (16–40 μM SH/g protein) which were determined by Wolosiak and Klepacka [2002].

In order to evaluate the molecular weights and to separate particular fractions, the protein were analyzed by means of exclusion chromatography in HPLC system (Table 2).

In all protein separations, fraction with the shortest retention time (peak 1) was eluted along with the void volume of the column. Other protein fractions were separated, although the molecular weight of the second subunit could not be determined on a base of the calibration curve, because it appeared out of the column working range. Basing on the literature references [Sathe et al., 1984; Derbyshire et al., 1976], the first peak was identified as globulin 11S with molecular weight of 360 kDa in the case of bean preparations, while 330 kDa for broad bean and pea preparations; the second peak corresponded to globulin 7S (150 kDa).

Analysis of separations revealed quite great similarity between broad bean and pea proteins. It was found that a subunit with MW 330 kDa (peak 1) dominated. Globulin 11S made up about 43% of all broad bean proteins, while in pea proteins separations, it was eluted along with globulin 7S making up 72.5% proteins. In broad bean proteins, subunit with MW 150 kDa (peak 2) was the second – referring to the peak area surface – fraction (about 20%). Bean proteins, unlike those at pea and broad bean, contained much less globulin 11S (5.5–8.5%) with molecular weight amounting to 360 kDa (peak 1). Globulin 7S (so-called phaseolin, peak 2) making up from 33.5 to 59.5% proteins was the main fraction in

these preparations. The runner bean var. Piękny Jaś tyczny was the exception, in which besides globulin 7S, also low-molecular fraction (about 30 kDa) identified as globulin 2S was present at significant quantities – 28.5% [Kulka and Grzesiuk, 1978]. Globulins with constant sedimentation 2S were also present in broad bean, pea, and other bean varieties proteins, although their shares were 12, 9.5, and 16.5-19%, respectively.

Table 2

Participation of the fractions of protein preparations determined by SE-HPLC

No.	MW [kDa]	Kind of preparation					
		BP	PP	WBP-PJT	WBP-P	BBP	RBP
		Participation (%)					
1	360	nd*	nd	5.5	6.5	8.5	6.0
2	330	**43.2**	**72.5**	nd	nd	nd	nd
3	150	**19.7**		**33.5**	47	**59.5**	**50.5**
4	55	8.8	13.0	nd	6.5		6.0
5	47.9	nd	nd	10.5	nd	nd	nd
6	33.6	12.0	9.5	**28.5**	nd	nd	nd
7	32.5	nd	nd	nd	**19.5**	**19.0**	**16.5**
8	18.6	1.7	2.0	nd	nd	nd	nd
9	16.2	4.7	3.0	10.5	6.0	5.5	6.0
10	13.5	nd	nd	4.0	3.0	2.5	2.5
11	12.4	nd	nd	3.5	3.5	2.0	3.5
12	11.3	3.8	nd	nd	2.5	nd	2.0
13	10.8	6.1	nd	nd	nd	nd	4.0
14	10.3	nd	nd	4.0	nd	1.5	nd
15	9.7	nd	nd	nd	5.5	1.5	3.0

nd * – not detected

Study results achieved by Carbonaro et al. [1997], that indicated the presence of remarkable quantities of high-molecular fractions (30% globulin 11S, 20% globulin 7S) in broad bean proteins, as well as about 40% share of fraction with MW 170 kDa corresponded to globulin 7S, were consistent with here achieved data for studied preparations.

On a base of chromatographic separations it can be suggested that proteins from preparations made of studied legume seeds are built mainly from globulin fractions. It results both from the seed composition, and the manner the preparations were produced (protein precipitation at pI). The protein isolation process at pI makes a reduction in albumin protein amount, because they are soluble in that pH range (4.2–4.3) and remain in a supernatant. It was confirmed by results achieved by Musakhanian and Alli [1987] for bean proteins. Those authors, when separating the alkaline protein preparations, found the presence of albumin fraction that was not observed in a sediment precipitated at isoelectric point. Globulin 7S was the dominating fraction that made up 82% of isolated proteins, as similar as in the case of here studied bean preparations.

The protein's surface hydrophobicity largely influences on functional properties of protein preparations, but their ability to adsorb on a non-polar phase surface may also play an important role in inhibiting the oxidation process due to antioxidant capacity of aromatic amino acids [Chen et al., 1996].

The protein hydrophobicity of three common bean varieties was at similar level (132-152 FI/% protein), although the lowest among tested preparations (Table 1). The broad bean proteins were characterized by slightly lower surface hydrophobicity (181 FI/% protein) than pea proteins (229 FI/% protein). Almost twice as high as for broad bean, and over 2.5-fold higher as compared to other bean varieties, hydrophobicity value (363 FI/% protein) was recorded for protein preparations of runner bean var. Piękny Jaś tyczny.

Achieved results revealed that globulins of examined preparations made of broad bean, pea, and bean seeds indicated low surface aromatic hydrophobicity, which was confirmed by studies upon the pea proteins performed by Gueguen [1989], in which also two main globulin fractions (vicilin and legumin) had low surface hydrophobicity. Values of surface aromatic hydrophobicity for globulins at bean, pea, and broad bean were slightly lower than those recorded in earlier studies [Worobiej et al., 2008] upon milk whey proteins (388-460j.u. FI/% protein), whose relatively low hydrophobicity at native state resulted from the fact that hydrophobic fragments were hidden inside the protein molecules [Pagliarini et al., 1990]. The protein surface hydrophobicity depends on their structure and aromatic amino acids availability in protein molecule greatly determines it. The literature data indicate the globulin 11S is characterized by higher hydrophobicity than globulin 7S [Gueguen, 1989]. The differences in protein hydrophobicity of tested preparations resulted from the shares of 11S and 7S fractions as well as low-molecular subunits (2S). The SE-HPLC protein analysis revealed (Table 2) that globulin 7S dominated in bean preparations, while globulin 11S in broad bean and pea preparations, which made that common bean proteins were characterized by lower hydrophobicity than broad bean and pea preparations. Proteins at runner bean (Piękny Jaś tyczny) contained significant quantities of low-molecular globulins 2S, which affected their highest surface hydrophobicity.

The hydroxyl radicals, as the most reactive oxygen form, initiate a variety of oxidation transformations within biological systems. Determining the abilities of tested preparations to scavenge the hydroxyl radicals is thus important at evaluating their efficiency as antioxidants.

Figure 1 presents results from determination of antiradical activity for studied legumes preparations against hydroxyl ($^{\bullet}$OH) radicals. Efficiency of $^{\bullet}$OH scavenging by protein preparations made of bean was high ranging within 77–90%. The lowest activity in deactivating the free radicals by these seeds preparations was observed in the case of runner bean var. Piękny Jaś tyczny, while the highest – for white bean var. Prosna. Preparations produced from color bean varieties deactivate the radicals at similar levels (84–86%). Slightly lower, although comparable to bean var. Piękny Jaś tyczny, efficiency of $^{\bullet}$OH radicals scavenging efficiency was manifested by broad bean (75%) and pea (78%) protein preparations. Achieved differences in antiradical activity against $^{\bullet}$OH radicals for common bean preparations, as compared to those made of other studied legumes, were statistically significant ($\alpha = 0.05$).

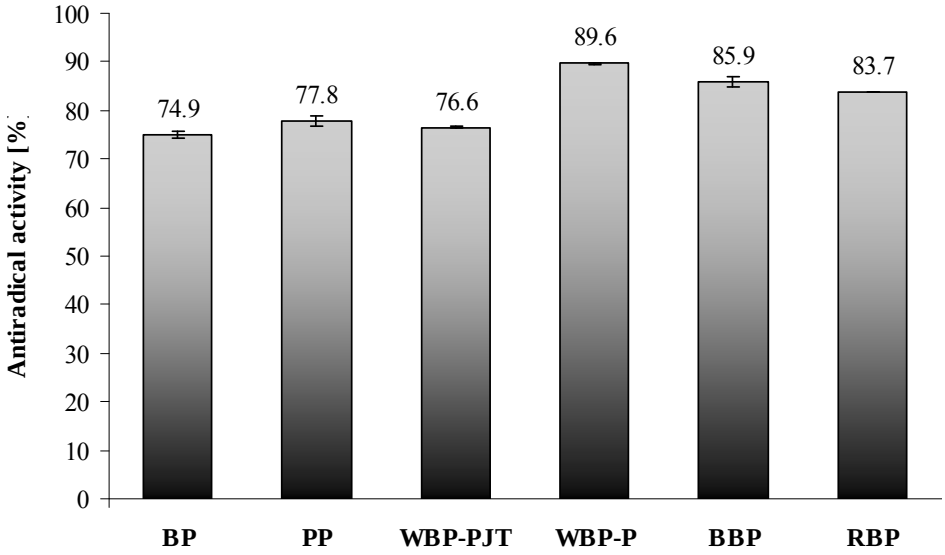

Fig. 1. Antiradical activity of protein preparations towards hydroxyl radicals

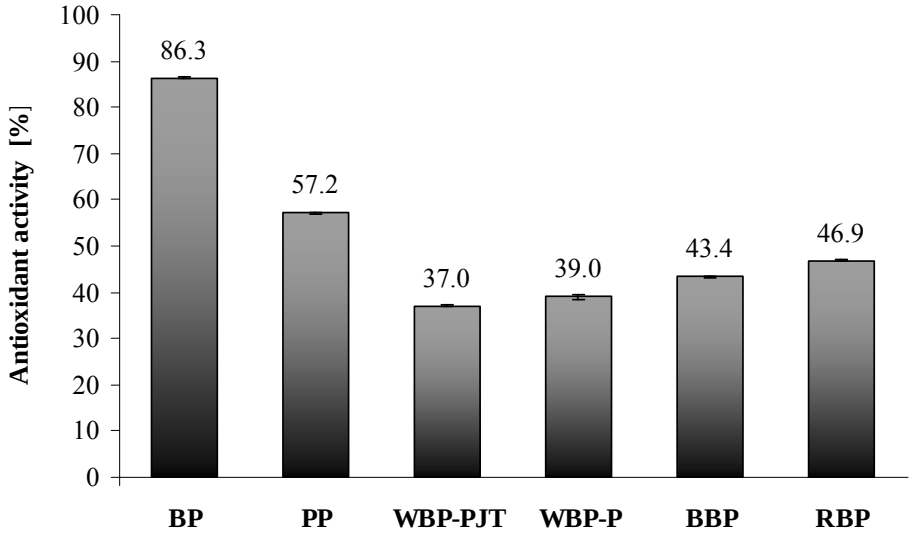

Fig. 2. Antioxidant activity of the protein preparations towards linoleic acid peroxides

Above results indicate that tested protein preparations, namely made of bean seeds, are effective scavengers of hydroxyl radicals. Higher antioxidant capacity of bean preparations may also result from larger number of histidine moieties in these seeds than in broad bean or pea globulins [Bhatty, 1982]. Histidine indicates the ability to complex Cu^{2+} ions – catalyst of the reaction producing the $^{\bullet}OH$ radicals in applied oxidation system [Chen et al., 1995]. Moreover, a positive correlation ($\alpha = 0.01$) between availability of thiol groups in protein preparations and their activity against hydroxyl radicals was found; its value was 0.95.

Wołosiak and Klepacka [2001] reported worse results of antiradical activity for albumins in legume preparations. The albumin preparations of pea (66 and 75%) showed better ability to scavenge the hydroxyl radicals than bean seed preparations (38 and 55%).

The antioxidant capacity of protein preparations in linolic acid emulsion was evaluated by means of determination of peroxides formed due to reaction catalyzed by hemoglobin addition. On a base of presented data (Figure 2), it is apparent that broad bean protein preparation was the most efficient (86%).

The bean protein preparations caused the inhibition of peroxide production at the level below 50%, although the lowest activity was found for white bean var. Piękny Jaś tyczny and var. Prosna (37 and 39%, respectively). It is worth mentioning that pea preparation was characterized by better antioxidant capacity (57%). The statistical analysis of results revealed significant differences between antioxidant activity of preparations made of legume seeds (broad bean, pea, and bean) in reaction of linolic acid oxidation. The result analysis indicated the lack statistically significant correlations ($\alpha = 0.1$) between aromatic hydrophobicity of tested protein preparations and their antioxidant properties in a system with linolic acid. The occurrence of aromatic amino acids in peptides, which makes contact with fatty acids easier, may cause their better antioxidant capacity [Chen et al., 1995].

Conclusions

Referring to results upon the ability to scavenge the free hydroxyl radicals, it can be summarized that common bean preparations showed the best antiradical activity, in which globulin 7S dominated, while broad bean and pea preparations with dominating globulin 11S, were the most efficient in protecting against the linolic acid oxidation. Preparation made of runner bean var. Piękny Jaś tyczny was characterized by the worst antioxidant capacity.

A correlation between number of available thiol groups and antiradical activity was found, whereas no such dependence was observed in the system with linolic acid. No influence of globulin surface aromatic hydrophobicity on antioxidant capacity was recorded.

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4

THE ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF HEN'S EGGSHELL PROTEIN EXTRACTS

Introduction

The main function of the egg is to provide nutritive substances required by the developing embryo as well as its protection against the outside environment [Mine and Kovacs-Nolan 2006; Trziszka 2000]. Numerous biologically active components, including antibacterial, antiviral, immunomodulatory, and anticancer factors, have been found in hen egg [Ahn and Ko 2004; Ball 2004; Froning 2004; Mine and Kovacs-Nolan 2006; Trziszka et al., 2006]. These components were mainly derived from egg yolk and egg albumen [Froning 2004; Mine and Kovacs-Nolan 2006]. There is currently increased focus on studying the bioactive components of eggshell [Mann et al., 2002; Wellman- Labadie et al., 2008a].

The chicken eggshell is a complex mixture of proteins and polysaccharides [Gautron and Nys 2007]. The organic material (2.5%) of the calcified layer includes 70% protein [Gautron and Nys 2007]. [Wellman-Labadie et al., 2008a; Mann et al., 2002]. The quality and quantity of the eggshell proteins differ among bird species and depends on their origin [Gautron and Nys 2007; Wellman-Labadie et al., 2008ab; Mann et al., 2003]. There is a group of unique proteins specific only to chicken eggshell. The main one of them, ovocleidin, occurs in at least two forms [Wellman-Labadie et al., 2008a]. This protein is synthesized in the tubular gland cells of the uterus and secreted into the uterine fluid in relative abundance during the calcification growth phase [Gautron and Nys 2007; Mann et al., 2002]. The major form of OC-17 is a phosphoprotein with two phosphorylated serines [Mann 1999]. A minor form is a glycolsylated 23-kDa protein with a C-type lectin domain which binds simple sugars [Mann 1999; Gautron and Nys 2007; Wellman-Labadie et al., 2008a]. Purified OC-17 is bactericidal against the Gram-positive bacteria *Bacillus subtilis, Staphylococcus aureus*, and *Pseudomonas aeruginosa* [Wellman-Labadie et al., 2008b]. A minor eggshell protein is ovocalyxin-36 (OCX-36), which showed significant identity with lipopolysaccharide-binding proteins (LBPs), bactericidal/permeability increasing (BPI) proteins, and the PLUNC family of proteins. As these proteins are described as "first-line host defense proteins", OCX-36 would contribute to the natural egg defense [Gautron and Nys 2007]. Another minor eggshell protein, ovocalyxin-25, contains two domains with significant identity to those found in serine protease inhibitors. Their role in the natural defense against microorganisms is unclear, but they could interfere with bacterial proteases necessary for pathogens' adhesion to hosts cells [Gautron and Nys 2007]. Proteins originally characterized in the egg albumen, such as lysozyme, ovalbumin, avidin, ovotransferrin, and ovomucoid, have also been observed in eggshell [Gautron and Nys 2007; Wellman-Labadie et al., 2008a]. They are known as a protective barrier against microorganisms. Fur-

thermore, low levels of gallinacin-8, β-defensin 11, as well as histones 2 and 4 have also been identified in the chicken eggshell matrix [Wellman-Labadie et al., 2008b]. Histones H1 and H2B demonstrate antimicrobial activity against both Gram-positive and Gram-negative bacteria [Wellman-Labadie et al., 2008b]. Mann et al. [2003] demonstrated antimicrobial activity of extracted proteins from demineralized eggshell against *Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Escherichia coli*, and *Salmonella entiriditis*. The recent mass spectrometric (MS) analysis of organic matrix components of eggshell allowed the identification of many new proteins, called ubiquitous proteins, which are widely expressed in various organs and milieus [Mann et al., 2008]. A final number of 520 proteins has been identified in the acid-soluble organic matrix of eggshell [Mann et al., 2008].

The propose of the present study was to evaluate the antimicrobial and antioxidant activities of eggshell protein extracts which may find application as a functional food ingredient.

Materials and methods

Biological Material. The eggs were laid by hens of the "green-legged" line. Only fresh eggs were analyzed.

Eggshell protein extraction

Acetic-acid extract [Mann and Siedler, 1999]. Fresh whole hens' eggs were broken and the albumen and yolk were separated from the shells. The shells were washed in bi-distilled water and incubated with 5% EDTA at 4°C for 30 min. Then the shells were separated from the shell membrane, washed in bi-distilled water, and dried. The eggshells were ground to a fine powder. Then 10% acetic acid was added (20 ml/g). After 24 h of incubation at 4°C the extract was centrifuged (14,000 x g, 4°C, 1 h) and dialyzed in bi-distilled water for 48 h (the water was exchanged 4 times). The obtained extract was lyophilized.

Ethanol extract. Eggshell powder was obtained as in the procedure for the acetic-acid extract. Then 40% ethanol and 10% acetic acid were added (10 ml/g). After 18 h of incubation at 4°C the extract was centrifuged (10,000 x g, 4°C, 30 min) and dialyzed against bi-distilled water for 48 h (the water was exchanged 4 times). The obtained extract was lyophilized.

Urea extract [Wellman-Labadie et al., 2008]. Whole eggs were washed in bi-distilled water and dried. Then the eggs were plunged into 1 M urea and incubated at room temperature for 20 min. The obtained extract was dialyzed against bi-distilled water for 48 h (the water was exchanged 4 times) and lyophilized.

Hydrochloric-acid extract [Wellman-Labadie et al., 2008b]. Whole eggs were washed in bi-distilled water and dried. Then the eggs were plunged into 1 M HCl and incubated at room temperature for 5 min. The obtained extract was dialyzed against bi-distilled water for 48 h (the water was exchanged 4 times) and lyophilized.

Determination of the molecular weight distribution of the eggshell protein extracts

The molecular weights of the eggshell protein extracts were determined by gel chromatography on a Zorbax GF-250 (4.6 × 250 mm, Agilent) column. The elution buffer was composed of 0.2 M phosphate buffer and 0.2 M NaCl (pH 7.0). The flow rate was 0.5 ml/min and the absorbance was monitored at 230 and 280 nm. The column was previously standardized using the molecular mass standards ribonuclease A (13.7 kDa), β-lactoglobulin (18.4 kDa), egg albumin (45 kDa), and lysozyme (14.4 kDa).

Protein assay

The protein concentration was determined according to the method of Lowry et al. [1951]. A standard curve was prepared for bovine serum albumin (BSA).

Amino-acid content determination

Samples (10 μg) were dried and hydrolyzed in 6 M HCl containing 1% phenol. After three cycles of N_2 purging and evacuation, the samples were placed in a hydrolysis oven and left at 115°C for 24 hours. Amino-acid derivatization was accomplished by adding a freshly prepared solution of 10% PITC (ethanol: water: TEA (triethylamine): PITC (phenylisothiocyanate) 7:1:1:1:1(4 things, 5 numbers) and leaving it for 20 min at room temperature. Then the phenylthiocarbamyl derivatives were separated on a PicoTag 3.9 x 150 mm column (Waters, Milford, MA, USA) in an HPLC system. The column was calibrated with three different concentrations of an amino-acid standard composed of 23 pure amino acids (Pierce USA).

Antioxidant capacity as scavenging of the free radical 1,1diphenyl-2-picrylhydrazyl (DPPH)

The antioxidant capacity was measured according to the method described by Yen & Chen [1995] with slight modifications. The tested samples were dissolved in water to a final volume of 1 ml and mixed with 1 ml of ethanol (98%). The reaction was started by adding 0.5 ml of 0.3 μM DPPH in ethanol. The mixtures were left for 30 min at room temperature and the absorbance of the resulting solution was measured at 517 nm. Radical scavenging activity of the peptides was expressed as μM Trolox/mg protein by reference to a standard curve.

Antimicrobial activity

Diffusion method. The strains *Yersinia enterocolitica*, *Listeria monocytognes, Enterococcus faecalis*, *Salmonella enteritidis*, and *Salmonella typhimurium* were grown in brain heart infusion (BHI) medium at 37°C for 18 h. The inocula were diluted to a final concentration of approximately $1x10^6$ CFU/ml. In the nutrient plates with BHI. 5-mm-diameter wells were punched and filled with 15 μl of extract solution with a concentration of 3 mg/ml or ampicillin as a control. Each bacterial suspension was spread over the surface of BHI agar as a thin film. The plates were incubated at 37°C for 24 h and the growth inhibition zones were observed.

Broth medium. [Pellegrini et al., 2004]. The strains *E. coli* PCM 2560, *B. subtilis* B172, *B. cereus* B3p, *B. laterosporum* B6, *B. cereus* B512, and *B. subtilis* B3 were tested. The protein extracts (1 mg/ml of cultivation medium) were mixed with bacteria harvested at the logarithmic phase (ca 1 x 10^4 CFU/ml) and incubated in trypticase soy broth (TSB) at 37°C for 2 h. The bacterial suspensions were diluted and plated on trypticase soy agar (TSA) for CFU evaluation. The assays were conducted in triplicate. The antibacterial activity was calculated as log (N_0/N_K), where N refers to the number of bacterial colonies after 2 h of incubation grown without (N_0) and with (N_K) the protein extracts.

Statistical analysis

All assays were conducted in triplicate. The results were analyzed using the Statistica 7.0 Analysis of Variance (ANOVA) program. Significance was set at the level of $p \leq 0.05$.

Results

Extraction

The extracts of the eggshell proteins were prepared in two ways. The acetic-acid and ethanol extracts were obtained by incubating dry, purified, ground eggshells with the particular solvent. In preparing the hydrochloric-acid and urea extracts, whole eggs were used. The obtained solutions of washed-out proteins were dialyzed against water. The concentrations of protein were determined in the preparations. Significant differences in protein concentration were noted among all the extracts. The most efficient solvent appeared to be hydrochloric acid (1 M), whereas the amount of protein obtained in urea solution (1 M) was about 56% lower. Acetic acid (10%) and ethanol (40%) were remarkably less potent in obtaining eggshell-derived proteins. The efficiency of extraction in these cases was more than 98% lower than in hydrochloric acid. In further tests, standardized extracts with a protein concentration of 1 mg/ml were used.

Table 1

Total protein concentration in the extracts obtained from 100 g of eggshell*

Extract	STEP	Total protein [mg]	Efficiency [%]
Acetic acid	raw	272	100
	dialyzed	79	29
Ethanol	raw	143	100
	dialyzed	130	91.5
Hydrochloric acid	raw	14,530	100
	dialyzed	12,932	89
Urea	raw	6354	100
	dialyzed	5782	91

*The weights of eggshell in the urea and hydrochloric-acid extracts were estimated by assuming a 12% share of eggshell in egg weight

Determination of the molecular weight distribution of the eggshell protein extracts

The molecular weight distributions of the proteins present in the eggshell extracts were characterized using size-exclusion chromatography with a separation range of 4.0 to 900 kDa. It has been shown that the contribution of proteins within the particular range of molecular masses was different in each extract (Tab. 2). In the ethanol, urea, and acetic-acid extracts, small proteins and peptides with molecular masses below 12 kDa (about 93%, 82, and 73% of total protein, respectively) prevailed, whereas in the hydrochloric-acid extract the dominant group was proteins with molecular masses in the range of 43–47 kDa (39%) and the smallest proteins constituted only about 20% of the total protein. Proteins with molecular masses from 12 to 17 kDa were observed in all the extracts, although at different concentrations (about 24, 13.4, 11.5%, and 6.1% in the hydrochloric-acid, urea, acetic-acid, and ethanol extracts, respectively). Components with molecular weights of 22–26 kDa were detected only in the acetic-acid and urea extracts (3.3 and 1.2%, respectively). The greatest variety in component size was noted in the acetic-acid extract, in which proteins in all the investigated ranges of molecular weight were observed. It was also the only extract in which molecules with masses of about 78–82 and 114–120 kDa were indentified.

Table 2

Molecular mass distribution of proteins in the eggshell extracts

Extract	Relative content of proteins (%) with molecular masses in the ranges (kDa)						
	114–120	78–82	43–47	32–36	22–26	12–17	< 12
Acetic-acid extract	1.2	3.0	4.1	4.2	3.3	11.5	72.7
Ethanol extract	–	–	1.0	–	–	6.1	92.9
Hydrochloric-acid extract	–	–	39.0	15.0	–	24.3	21.7
Urea extract	–	–	–	3.1	1.2	13.4	82.3

Amino-acid composition

The amino-acid composition of the proteins in all the extracts was analyzed. As shown in Tab. 3, the amino-acid compositions of the extracts differed significantly. In the acetic-acid extract, the highest concentrations of acidic amino acids such as Glu/Gln (19%) and Asp/Asn (14%) were noted, whereas in the hydrochloric-acid extract the content of these amino acids was the lowest of all the extracts (about 5.5 and 2%, respectively). In the urea extract, small hydrophobic amino acids such as glycine (24%) and alanine (10%) as well as serine (17.5%) prevailed. The extracts also differed in their content of proline. The largest amount of this amino acid was found in the ethanol extract (10%), slightly lower in the hydrochloric-acid extract (9%), and significantly lower in the acetic-acid and urea extracts (5 and 3%). Much different was also the amount of arginine, of which the highest concentration was observed in the hydrochloric-acid extract (8.5%) and the lowest in the urea extract (2.5%). Methionine was detected in the proteins of the hydrochloric and urea extracts.

Table 3

Amino-acid composition of eggshell protein extracts (mol%)

Amino-acid residue	Acetic-acid extract	Ethanol extract	Hydrochloric-acid Extract	Urea extract
Asp/Asn	14.10	7.05	1.96	7.71
Ser	13.77	9.59	8.44	17.48
Glu/Gln	19.23	13.67	6.53	18.03
Gly	8.95	13.34	18.85	24.09
His	2.35	1.84	2.68	0.95
Arg	5.46	7.94	8.43	2.51
Thr	6.01	5.26	7.13	5.31
Ala	5.99	8.75	9.97	10.27
Pro	5.01	9.96	8.85	3.41
Tyr	0.96	0.93	2.67	3.34
Val	5.80	8.17	8.48	4.98
Met	0.00	0.00	1.76	1.72
Lys	2.98	3.27	3.83	0.45
Ile	2.72	2.87	3.44	2.20
Leu	4.75	5.67	5.10	5.20
Phe	1.93	1.69	1.89	2.85

Antimicrobial activity

The antimicrobial activity against food spoilage bacteria (Fig. 1) as well as against pathogenic bacteria (data not shown) was investigated. Significant differences were observed among the investigated preparations. The highest antimicrobial activity was noted when the hydrochloric-acid extract was applied against *Bacillus* species. It caused a reduction of about 0.33–0.84 (log N_0/N_k). Only *B. cereus* B 512 was resistant to the action of the hydrochloric-acid extract. Bacillus strains, except for *B. subtilis* B3, were also sensitive to the urea extract. The acetic-acid extract exhibited antimicrobial activity against the Gram-negative *E. coli* PCM 2560. The Gram-positive bacterium *B. subtilis* B 172 was also susceptible to the action of this extract, whereas it had no reducing effect on the other investigated bacterial species. The ethanol extract showed a very narrow spectrum of bactericidal activity which was limited to *B. laterosporum* B6. None of the protein extracts had any effect on *Yersinia enterocolitica, Salmonella enteritidis, Salmonella typhimurium, Listeria monocytognes*, or *Enterococcus faecalis*.

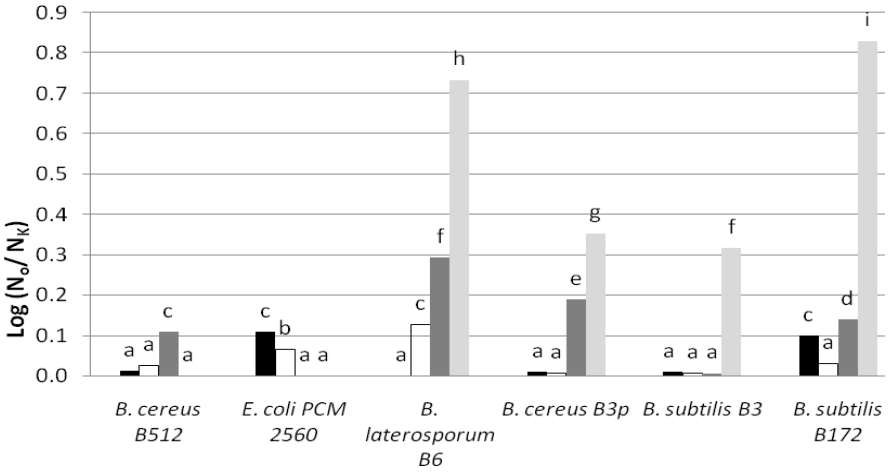

Fig. 1. Antimicrobial activity of the protein extracts derived from hen eggshell. ■ Acetic-acid extract, □ Ethanol extract, ■ Urea extract, ■ Hydrochloric-acid extract.

Antioxidant capacity

The antioxidant activity of all the extracts were monitored by determining their radical scavenging capacity (Fig. 2). The activity was expressed as the trolox equivalent. The hydrochloric-acid and urea extracts exhibited significantly higher antioxidant activity (0.27 and 0.2 μM trolox/mg, respectively) than the ethanol and acetic-acid extracts (0.01 and 0.03 μM trolox/mg, respectively).

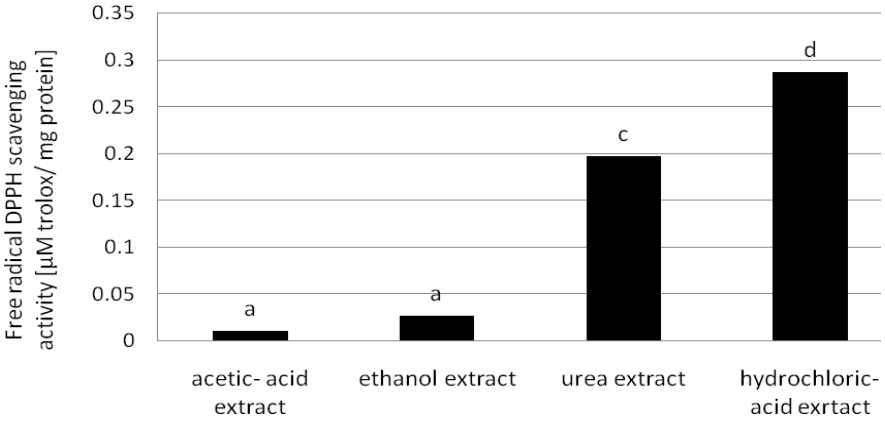

Fig. 2. Antioxidant activity expressed as free radical DPPH scavenging capacity of the eggshell protein extracts

Discussion

The chicken egg is an important source of nutrients, containing all the proteins, lipids, vitamins, minerals, and growth factors required by the developing organism as well as a number of defense factors to protect against bacterial and viral infection. Moreover, egg albumen and yolk are well known as a source of biologically active substances with specific benefits for human and animal health and with possible applications in medicine, pharmacy, and the food industry. Much less is known about the properties of eggshell proteins. In our study we investigated four different preparations of eggshell-derived proteins, with special focus on their antimicrobial and antioxidant activities.

It was observed that the kind of solvent used for protein extraction highly affected protein quantity and the quality of the final extract. Hydrochloric acid was the most efficient solvent of eggshell proteins, although more variable material was detected in the acetic-acid extract. Based on molecular weight, the proteins present in the extract may correspond to ovocalyxin (116 kDa), ovotransferrin (76 kDa), ovalbumin (45 kDa), ovocalyxin (32 kDa), ovocalyxin (34 kDa), ovoleidin (23 kDa), ovocleidin (17 kDa), and lysozyme (14.4 kDa). This is in line with results obtained by other authors. Total water-soluble proteins obtained after chicken eggshell demineralization in 10% acetic acid were extensively studied by Mann et al. [1999, 2002]. They also identified several proteins in the acetic-acid extract of hen eggshells. Of these, the main components were OC-17, ovalbumin, ovotransferrin, and OCX-116. However, the protein extract obtained under the same conditions by Wellman-Labadie et al. [2008a] contained mainly OC-17, ovalbumin, and OCX-116. Further separation of the acetic-acid protein extract by RP-HPLC indicated the presence of the main proteins and the mixture contained many fragments of the eggshell proteoglycan core protein ovocleidin-116 [Mann et al., 2002]. These authors suggested that fragmentation of ovocleidin-116 may occur during extraction of the matrix proteins. In many fragments, amino-acid residues such as Ile, Arg, and Phe were observed on the N-terminus, which indicated that thrombin-like and chymotrypsin-like protease activities were present in the eggshell extracts. Proteins and peptides with molecular masses below 12 kDa constituted the main group of eggshell-derived products in all our preparations except for the hydrochloric-acid extract.

While protein extraction of hen eggshell by acetic acid is widely used, there is not much information about extraction methods with hydrochloric acid, urea, or ethanol. HCl and urea were used for extracting eggshell proteins of various bird species, such as the Canada Goose, Mute Swan, Wood Duck, and Hooded Merganser [Wellman-Labadie et al., 2008b]. As the quality of proteins in hydrochloric-acid and urea extracts varied across the species, we cannot directly compare them with our hen eggshell extracts. However, HCl treatment of various species' eggshells led to an extract with higher amounts of lysozyme, whereas urea treatment resulted in a very low concentration of this protein. This is comparable with our results. We obtained the highest concentration of proteins with molecular masses of 12-17 kDa (corresponding to lysozyme and OC-17) when the hen eggshells were extracted with hydrochloric acid.

To the best of our knowledge, this is the first report using ethanol in hen eggshell protein extraction. However, it is well known that ethanol causes the precipitation of proteins of higher molecular weight, whereas small proteins, such as lysozyme, are more resistant to precipitation by this agent [Sokołowska et al., 2007]. Under these conditions, we detected

mainly proteins with molecular masses below 17 kDa in the supernatant. The differences in protein composition among the extracts was confirmed by the amino-acid composition. The presence of methionine and a high concentration of proline were observed in the ethanol and hydrochloric-acid extracts.

It is well known that the presence of definite amino-acid residues in the primary structures of proteins and peptides enhance their biological activity. For example, peptides and proteins rich in Arg and Trp residues have broad and potent antimicrobial activity. Trp has a distinct preference for the interfacial region of lipid bilayers, while Arg residues endow the peptides with cationic charges and hydrogen-bonding properties necessary for interaction with the abundant anionic components of bacterial membranes [Chan et al., 2006]. The highest concentration of Arg residues was observed in the amino-acid composition of the eggshell proteins obtained by hydrochloric-acid treatment. This extract also exhibited considerable antimicrobial activity against Bacillus species, especially *B. laterosporum* B6 and *B. subtilis* 172.

The antioxidative activity of proteins and peptides depends on their amino-acid sequences as well as the constituent amino acids. His, Leu, Pro, and Tyr residues are preferred for potential antioxidant activity [Chen et al., 1996]. The presence of these amino acids in eggshell protein extracts may also exhibit antioxidant activity. In our study, the hydrochloric-acid extract, whose amino-acid composition showed a considerably high concentration of His, Pro, and Tyr compared with the other extracts, exhibited the most remarkable capacity for free radical scavenging. The material extracted from eggshell by urea, in which the highest level of Tyr was determined in the amino-acid composition, also exhibited relatively high antioxidant activity. In the amino-acid composition of the proteins present in both the hydrochloric acid and urea extracts, methionine, which can be involved in oxidation, was also detected.

Food spoilage and pathogenic bacteria activity and their growing resistance to conventional preservatives as well as the lipid oxidation of food components are major problems in the food industry. Therefore, bifunctional natural proteins or peptides with both antimicrobial and antioxidant activities could be considered ideal candidates for use in the food industry.

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5

ANTIOXIDANT ACTIVITY OF ROSE LIQUEURS

Introduction

Liqueurs made from fruit or herbs became more popular recently. They are traditional beverages produced in Poland for centuries. They were initially used to treat pain and as a digestive aid and later served as an aperitif. Liqueurs are usually made from plant material, naturally rich in phenolic compounds which play an important role in taste and colour creation.

Food with high antioxidant potential is gaining increasing popularity. The come back to the old and forgotten traditional products is observed, and liqueurs are in this group. One of the most popular liqueurs in eastern part of Poland was Żenicha Kresowa, prepared by girls before consider as ready to marriage.

Rose liqueurs are very attractive products because of fruit, one of the richest source of flavonoids, responsible for most of antioxidant activity.

Among wild fruit growing in Poland rose fruits deserve special attention due to high concentration of vitamin C, carotenoids, organic acids, carbohydrates and minerals. As mentioned, they are rich in flavonoids and some species in antocyanins as well.

Liqueurs, like wines are products containing fruit constituents which are extracted in ethanolic solutions. They are characterized by a specific taste, aroma and colour. During aging the constituents and colour undergo changes and liqueurs obtain right balance of taste.

There is a large number of recipies for the production of liqueurs. Since the production is easy, the process differs in grade of ethanol used, time of fruit extraction and sequence of sugar and/or ethanol adding.

Antioxidant activity of wines and others processed fruit was described by many researchers [Czyżewska and Pogorzelski, 2002; Kivilompolo et. al., 2008; Lodovici et. al., 2001; Netzel et. al., 2003]. Relatively little information is yet known about the content of phenolic compounds and antioxidant activity in these kind of beverages, which can be made from different fruit and herbs. Hence, fruit liqueurs may be good alternative to wines and others alcoholic beverages.

The aim of this work was to investigate the antioxidant activity and colour of liqueurs made from four species of rose fruit by addition of 45° or 65° grade ethanol and sugar on different stages of preparation and evaluation of antioxidant activity changes during seasoning.

Materials and methods

The liqueurs were prepared in 2007 year from frozen fruit of four Rosa sp.: *R. rugosa, R. canina, R. spinosissima* oraz *R. hybrida* picked up near Wrocław.

Sample preparation:

The liqueurs were prepared in four variants: with the use of ethanol of 45% and 65% v/v grade, from fruits with and without seeds, and with the addition of sugar on various stages of the product preparation (at the beginning and after 1 month of ethanolic extraction).

Frozen fruits (100 g) with or without seeds were put into glass jar with ethanol (100 g) or/and sugar (50 g), depending on variant used, as given in Table 1. Extraction was kept at room temperature, in the dark for 14–30 days. Liqueurs were aged 3, 6, 9 and 26 months at ambient temperature i.e 23–28°C, without light.

Table 1

Variants of rose liqueurs preparation

Variant	*R. rugosa* and *R. canina*			Addition	
				after 14 days	After 30 days
V1	Whole fruit	–	Alcohol 45%	–	Sugar
V2	Whole fruit	Sugar	-	Alcohol 45%	–
V3	Whole fruit	Sugar	Alcohol 45%	–	–
V4	Whole fruit	Sugar	Alcohol 65%	–	–
	R. spinosissima and *R. hybrida*				
V1	Whole fruit	–	Alcohol 45%	–	Sugar
V2	Whole fruit	Sugar	-	Alcohol 45%	–
V1a	Fruit without seeds	–	Alcohol 45%	–	Sugar

Analytical methods:

The total polyphenols were determined by the Folin-Ciocalteu's method [Slinkart & Singleton, 1977]. The results were calculated as mg of gallic acid in 100 ml.

Anthocyanins were determined by the method of Fuleki and Francis [1968]. Ferric reducing antioxidant power (FRAP) was measured according to Benzie and Strain [1996].

DPPH assay was carried out as described by Yen and Chen [1995]. ABTS assay was done according to the method of Re et al. [1999]. Results of antioxidant activity are expressed in μM Trolox equivalents (μMT)/100 ml. Spectrophotometric measurements were made using Shimadzu UV-2401 UV-VIS spectrophotometer (Japan)

The color of liqueurs was measured using a Color Quest XE colorimeter (HunterLab, Hunter Associates Laboratory, Inc. , USA). L^*, a^*, b^*, chroma (C^*) and hue angle (h^o) were measured in triplicate in the total transmission mode using Illuminant D_{65} and 10^o observer angle.

Viscosity was measured on Brookfield viscosimeter DV-II (Brookfield Engineering Laboratories, Inc., USA), with 00 spindle and 50 rpm after 3 and 26 months.

Extract was determined by digital refractometer (ATAGO, Japan).
All determinations were performed in triplicate.
Analysis of variance was performed using Statictica 8.0 software.

Results and discussion

Changes in polyphenols and antioxidant activity

The liqueurs were obtained with different efficiency from 42 to 73% in comparison to ingredients used. Viscosity ranged from 6.02 to 45.80 mPas after 3 months and decreased during aging by about 50% (Table 2). Higher viscosity was observed in samples made from fruits extracted initially with ethanol with or without sugar.

Rose hips are well known, rich source of phenolic compounds. Their content may be different among species. In *R. hybryda* and *R. spinosissima* Babis i Kucharska [2004] determined 1610 mg/100 g and 1205 mg/100 g of phenolic compounds respectively. Oszmiański and Chomin [1993] reported, that total phenolic content in *R. rugosa* hips was 9500 mg/100 g. Similarly, Leja et al. [2007] determined 971 mg/100 g in *R. canina* fruit. Especially noteworthy are fruits of *R. spinosissima* due to its dark color. Anthocyanin content in fruits ranged from 300 to 550 mg/100 g [Babis and Kucharska, 2004].

Table 2

Viscosity and polyphenol contents in rose liqueurs

Liqueur	Variant	Viscosity mPas		Total polyphenols mg GAE/100 ml(± SD)							
		3	26	3		6		9		26	
R. canina	1	12.6	5.04	69.5	±0.4^{bc}	61.9	±4.9^{ef}	80.6	±1.9^{a}	60.6	±0.7^{f}
	2	10.8	3.74	62.9	±2.1^{def}	66.7	±0.6^{cd}	70.4	±1.1^{bc}	46.4	±0.0^{i}
	3	11	3.68	64.8	±0.2^{de}	64.3	±1.3^{def}	72.1	±0.6^{bc}	54.5	±2.7^{gh}
	4	17.7	8.65	56.4	±5.6^{g}	62.1	±1.3^{ef}	70.0	±0.7^{bc}	52.1	±0.3^{h}
R. rugosa	1	6.71	4.60	28.3	±1.8^{i}	42.4	±0.2^{d}	44.1	±0.7^{d}	33.3	±0.4^{h}
	2	5.47	1.99	49.0	±1.2^{c}	51.2	±1.6^{b}	53.4	±1.4^{a}	39.6	±0.6^{e}
	3	6.13	2.26	40.5	±0.6^{e}	33.1	±0.6^{h}	43.9	±0.9^{d}	33.2	±1.0^{h}
	4	7.35	3.02	35.5	±0.2^{g}	36.1	±0.0^{fg}	37.6	±0.0^{f}	29.7	±1.2^{i}
R. spinosissima	1	8.1	3.95	119.6	±0.7^{c}	113.5	±3.3^{cd}	133.8	±3.5^{b}	118.4	±2.0^{c}
	2	7.29	3.15	64.9	±5.7^{g}	94.8	±3.9^{f}	108.1	±0.3^{de}	99.6	±5.4^{f}
	1a	8.51	4.19	113.3	±4.8^{cd}	131.9	±5.7^{b}	148.2	±0.5^{a}	101.3	±4.7^{ef}
R. hybrida	1	45.8	26.00	59.6	±3.5^{d}	77.0	±1.5^{ab}	81.9	±1.1^{a}	47.7	±2.4^{e}
	2	6.02	nd	76.3	±7.4^{ab}	72.3	±4.0^{b}	65.5	±0.8^{cd}	49.4	±2.7^{e}
	1a	20.2	nd	69.8	±3.5^{b}	76.9	±0.0^{ab}	73.8	±2.7^{ab}	47.5	±3.2^{e}

Results are the means ± SD. Statistically homogeneous (p value ≤0.05) groups (within one fruit liqueurs) are designated with the same letters.

Liqueurs from *R. canina* and *R. rugosa* fruit were made in four variants and those from *R. hybrida* and *R. spinosissima* in three variants and V1 and V2 variants were made from all kind of rosa.

In samples prepared under wariant V1, highest content of total phenols was determined in *R. spinosissima* liqueurs (1200 mg GAE/100ml) and almost twice less (600–700 mg GAE/100ml) in *R. hybrida* and *R. canina* liqueurs. Antiradical activity against DPPH radicals on level 5000-6000 μMT/100ml in samples from *R. spinosissima* and *R. hybrida* was found. Liqueurs from *R. canina* and *R. rugosa* were almost three times less active. In the ABTS assay, liqueurs from *R. spinosissima* and *R. hybrida* exhibited higher activity (5156 μMT/100 ml and 3046 μMT/100 ml respectively) than the others.

Similarly to variant V1, among the samples made according to variant V2, the highest phenolic compounds content and antioxidant activity showed liqueurs from *R. hybrida* and *R. spinosissima*. These from *R. canina* and *R. rugosa* were two or more times weaker

A general observation relating to species of rosa fruit is that *R. spinosissima* liqueurs exhibited the highest antioxidant activity and total phenols levels, probably due to its anthocyanin content. In our investigation, anthocyans in liqueurs from *R. spinosissima* after 3 three months were from 20 to 29 mg/l. Heinonen et al. [1998] determined the total phenolic contents in fruit wines and liqueurs at a level of 16.0–182.0 mg/100 ml. In comparison, our liqueurs contained 30 to 170 mg of phenolics in 100 ml after 26 months of storage. Nocino liqueurs contained from 29 to 388 mg/100 ml [Alamprese et al., 2005]. White wines [Kallithraka et al., 2009; Paixao et al., 2007] contained 23–43 mg/100 ml, and red 65–193 mg/100 ml. It shows that liqueurs can be valuable sources of phenolic compounds.

R. spinosissima liqueurs were prepared in two variants, differing in the order of addition of alcohol and sugar and by means of fruit preparation (with or without seeds). Samples received from the variant V1, both from the whole fruit and without seeds, contained almost twice as many polyphenols (above 1100 mg/100 ml) than liqueurs prepared by variant V2, after three months of ageing. In variant V1 fruits were initially extracted by ethanolic solution and after 14 days sugar was added, so the phenols extraction in solutions without sugar was more efficient.

Table 3 shows antioxidant activity of liqueurs. It was observed that liqueurs activity against ABTS radicals and FRAP values were almost twice as high as against DPPH.

Storage of liqueurs up to 9 months resulted in increase of phenolic compound content, and after 26 months decrease was observed to values near initial. Activity against ABTS and DPPH radicals showed similar tendency. In liqueur V2 activity towards DPPH radical were higher than in V1 in 3 months time, but decreased almost three times after 26 months of maturation.

The method of *R. hybrida* liqueurs preparation had no influence on antioxidant activity and total phenolic content. During 26 months of storage, phenols content and activity against ABTS increased first, then decreased. Reducing power and DPPH activity decreased all the time of aging.

Similar fluctuations of phenolic compounds and antioxidant activity during fruit juices storage were observed by Piljac-Zegarac et al. [2009] and Klimczak et al. [2007] During storage some compounds are formed and enhance total phenolic content.

Table 3

Antioxidant activity of rose liqueurs

	Variant	Aging time [months]	DPPH	ABTS	FRAP
			µM T/100 ml(± SD)		
R. canina	V1	3	1701 ± 23^{b}	2199 ± 42^{i}	3742 ± 36^{bc}
		6	1544 ± 45^{c}	3944 ± 54^{a}	3848 ± 131^{b}
		9	1232 ± 09^{f}	3778 ± 81^{ab}	3612 ± 57^{c}
		26	1147 ± 25^{hg}	3355 ± 277^{cd}	2841 ± 11^{f}
	V2	3	1430 ± 00de	1889 ± 84^{j}	3321 ± 79de
		6	1170 ± 71^{g}	3191 ± 12de	2628 ± 103^{g}
		9	902 ± 14^{k}	2567 ± 62^{h}	2671 ± 31^{g}
		26	1458 ± 15^{d}	2903 ± 87^{fg}	4186 ± 62^{a}
	V3	3	1582 ± 10^{c}	2170 ± 217^{i}	3379 ± 27^{d}
		6	1480 ± 06^{d}	3701 ± 72^{b}	3205 ± 85^{e}
		9	1079 ± 69^{i}	3250 ± 73^{cde}	2938 ± 59^{f}
		26	1089 ± 33^{hi}	2950 ± 14^{fg}	2647 ± 99^{g}
	V4	3	1778 ± 48^{a}	1787 ± 84^{j}	2919 ± 70^{f}
		6	1389 ± 06^{e}	3454 ± 24^{c}	2848 ± 116^{f}
		9	1055 ± 18^{ij}	3091 ± 89^{ef}	2882 ± 78^{f}
		26	1002 ± 27^{j}	2752 ± 57^{gh}	2470 ± 07^{h}
R. rugosa	V1	3	1842 ± 61^{a}	1157 ± 12^{j}	1717 ± 170de
		6	1521 ± 00^{c}	2616 ± 54^{bc}	2110 ± 143^{bcde}
		9	1144 ± 18^{e}	2280 ± 103de	1897 ± 39^{cde}
		26	1816 ± 12^{a}	2358 ± 64^{d}	3091 ± 23^{a}
	V2	3	1580 ± 97^{bc}	2136 ± 325^{ef}	2674 ± 82^{ab}
		6	1523 ± 03^{c}	2940 ± 78^{a}	2278 ± 03^{bcd}
		9	1149 ± 17^{e}	2677 ± 47^{b}	2277 ± 92^{bcd}
		26	1014 ± 08^{f}	2626 ± 60^{bc}	3124 ± 1137^{a}
	V3	3	1642 ± 10^{b}	1595 ± 18^{h}	2235 ± 106^{bgde}
		6	1377 ± 23^{d}	2612 ± 12^{bc}	1854 ± 36^{cde}
		9	1138 ± 25^{e}	2079 ± 21^{f}	1917 ± 14^{cde}
		26	1525 ± 50^{c}	2449 ± 36^{cd}	3163 ± 46^{a}
	V4	3	1785 ± 00^{a}	1361 ± 96^{i}	1923 ± 18^{cde}
		6	1439 ± 90^{d}	2323 ± 72^{d}	1687 ± 255de
		9	1138 ± 67^{e}	1787 ± 60^{g}	1507 ± 20^{e}
		26	1838 ± 39^{a}	1888 ± 61^{g}	2480 ± 37^{abc}

Table 3 cont.

1	2	3	4			5			6		
R. hybrida	V1	3	6002	±	13^{a}	3046	±	313^{d}	3530	±	24^{b}
		6	4435	±	39^{b}	5054	±	24^{a}	3822	±	268^{a}
		9	2732	±	79^{d}	5060	±	227^{a}	3427	±	38^{b}
		26	1455	±	83^{e}	3164	±	185^{cd}	1804	±	109^{f}
	V2	3	5892	±	90^{a}	2876	±	168^{d}	4046	±	97^{a}
		6	3707	±	64^{cd}	4356	±	00^{b}	3461	±	730^{cd}
		9	1536	±	606^{e}	3698	±	71^{c}	2370	±	85^{e}
		26	1544	±	108^{e}	3011	±	49^{d}	1847	±	51^{f}
	V1a	3	5455	±	373^{a}	3318	±	217^{cd}	2773	±	146^{d}
		6	3880	±	773^{bc}	5003	±	289^{ab}	3203	±	195^{c}
		9	1730	±	203^{e}	1963	±	260^{e}	2252	±	59^{e}
		26	1479	±	100^{e}	3042	±	129^{cd}	2234	±	741^{f}
R. spinosis-sima	V1	3	5036	±	116^{c}	5156	±	72^{e}	6385	±	122^{b}
		6	5073	±	116^{c}	8117	±	24^{b}	5044	±	292^{e}
		9	4262	±	114^{e}	7204	±	98^{c}	5127	±	155^{de}
		26	3654	±	90^{f}	7299	±	144^{c}	5045	±	74^{e}
	V2	3	9981	±	26^{a}	3999	±	361^{f}	4923	±	24^{e}
		6	4453	±	219^{de}	6722	±	217^{cd}	4115	±	170^{f}
		9	3619	±	100^{f}	5287	±	208^{e}	3947	±	105^{f}
		26	2916	±	86^{h}	6169	±	95^{d}	3935	±	76^{f}
	V1a	3	5938	±	00^{b}	5054	±	217^{e}	7013	±	328^{a}
		6	5965	±	39^{b}	8883	±	241^{a}	5439	±	122^{c}
		9	4584	±	110^{d}	8111	±	730^{b}	5398	±	162^{cd}
		26	3144	±	76^{g}	6467	±	192^{d}	4005	±	133^{f}

Results are the means ± SD. Statistically homogeneous (p value ≤0.05) groups (within one fruit liqueurs) are designated with the same letters.

Liqueurs from *R. rugosa and R. canina* were prepared in four variants. The highest concentration of phenolic compounds in samples from *R. canina* was determined in variant V3a (fruits without stones, ethanol and sugar added simultanously), V3 and V1. After 9 months time content of polyphenols increased from 10 to 20%, and then decreased varied from10 to 35% in comparison to level determined after 3 months of storage, most of all in V2 (first fruit+sugar, alcohol after 14 days). The best results were observed in V1 liqueur and the worst in V2 liqueur.

Similarly, samples of *R. rugosa* liqueurs made according to the variant V2 contain the most total polyphenols, and those produced by V1 and V4 showed the highest antioxidant activity. Investigated features (properties) of *R. rugosa* and *R. canina liqueurs* changed in similar trends like in *R. hybrida* and *R. spinosissima,* but modifications observed were smaller.

Antioxidant activity and total polyphenols content of rose liqueurs was stable during aging. Similarly, Alampreese et al. [2005] and Stampar et al. [2006] showed that antioxidant activity of liqueur made with green, unripe walnuts (nocino liqueur) was directly correlated to the total phenol content and did not change during storage, even for many years.

Colour changes

The colour changes of all samples during storage is shown in Table 4.

Table 4

Colour parameters of rose liqueurs

	Variant	Aging time	L*	a*	b*	a/b	C*	h°	dL*	DE*
R. canina	V1	3M	71.1	15.7	78.3	0.2	79.8	78.7		
		6M	57.5	30.2	85.1	0.7	90.3	70.5	13.5	21.0
		9M	45.4	40.4	74.6	1.2	84.8	61.6	25.7	35.9
		26M	13.5	33.6	22.8	2.6	40.5	34.2	57.6	82.0
	V2	3M	66.1	19.3	79.5	0.3	81.8	76.4		
		6M	50.6	37.5	81.5	1.0	89.7	65.3	15.5	24.0
		9M	39.2	44.2	65.8	1.5	79.2	56.1	26.9	39.2
		26M	16.0	37.6	27.1	2.7	46.3	35.8	50.1	74.8
	V3	3M	77.7	8.7	69.5	0.1	70.0	82.8		
		6M	67.5	20.6	82.3	0.3	84.9	76.0	10.1	20.2
		9M	56.1	32.4	84.3	0.7	90.3	69.0	21.6	35.3
		26M	26.0	38.4	43.9	1.9	58.3	48.9	51.6	64.8
	V4	3M	77.2	9.7	70.1	0.1	70.7	82.1		
		6M	55.5	34.9	86.3	0.8	93.1	68.0	21.7	37.0
		9M	43.9	41.5	72.8	1.3	83.8	60.3	33.3	46.1
		26M	9.3	33.4	15.7	3.2	36.9	25.2	67.9	90.1
R. rugosa	V1	3M	76.9	10.3	72.6	0.1	73.4	82.0		
		6M	66.9	21.4	82.2	0.4	85.0	75.4	10.0	17.8
		9M	62.1	27.4	82.6	0.5	87.1	71.7	14.8	24.7
		26M	48.7	40.6	79.0	1.1	88.8	62.8	28.2	41.9
	V2	3M	60.1	26.0	82.6	0.5	86.6	72.5		
		6M	57.7	30.8	84.5	0.7	90.0	70.0	2.4	5.7
		9M	50.8	35.1	80.3	0.9	87.6	66.4	9.2	13.2
		26M	34.1	40.1	57.3	1.5	69.9	55.0	26.0	38.9
	V3	3M	77.3	9.3	71.6	0.1	72.2	82.6		
		6M	61.9	24.7	81.6	0.5	85.2	73.2	15.4	23.9
		9M	58.7	30.1	82.8	0.6	88.1	70.0	18.6	30.1
		26M	42.1	39.0	69.5	1.2	79.7	60.7	35.2	46.1
	V4	3M	74.1	10.2	70.0	0.1	70.8	81.7		
		6M	69.1	19.0	80.4	0.3	82.6	76.7	5.0	14.5
		9M	65.1	23.7	80.3	0.4	83.7	73.6	9.0	19.2
		26M	42.2	31.8	67.2	0.9	74.3	64.7	31.9	38.6

Table 4 cont.

1	2	3	4	5	6	7	8	9	10	11
R. hybrida	V1	3M	76.8	6.6	69.6	0.0	69.9	84.6		
		6M	61.0	16.5	75.4	0.3	77.2	77.7	15.8	19.5
		9M	49.9	22.1	73.3	0.5	76.5	73.2	26.9	31.3
		26M	30.6	42.8	51.6	1.9	67.0	50.4	46.1	61.4
	V1a	3M	82.9	3.4	69.8	-0.1	69.9	87.3		
		6M	64.6	27.5	92.5	0.5	96.5	73.5	18.3	37.8
		9M	38.6	39.6	64.4	1.4	75.6	58.4	44.3	57.5
		26M	26.1	45.0	44.2	2.3	63.0	44.5	56.8	74.9
	V2	3M	81.9	4.8	72.6	0.0	72.7	86.3		
		6M	69.2	22.2	90.9	0.3	93.6	76.3	12.7	28.3
		9M	49.5	38.1	80.5	1.0	89.1	64.7	32.4	47.2
		26M	27.1	44.8	45.6	2.3	64.0	45.5	54.8	73.1
R. spinosissima	V1	3M	13.5	42.7	22.9	3.7	48.5	28.2		
		6M	7.2	34.4	12.2	3.8	36.5	19.6	6.3	14.9
		9M	9.7	35.4	16.3	3.4	39.0	24.8	3.9	10.6
		26M	11.6	35.8	19.6	3.1	40.8	28.7	1.9	7.9
	V1a	3M	12.5	41.1	21.2	3.7	46.2	27.3		
		6M	11.0	38.0	18.6	3.5	42.3	26.1	1.5	4.3
		9M	10.2	36.2	17.1	3.5	40.0	25.3	2.3	6.8
		26M	6.3	29.5	10.4	3.4	31.2	19.4	6.2	17.1
	V2	3M	11.4	38.8	19.3	3.6	43.3	26.5		
		6M	7.7	34.1	13.0	3.7	36.5	20.9	3.7	8.6
		9M	13.1	38.7	22.0	3.3	44.5	29.6	-1.7	3.2
		26M	7.6	31.7	12.7	3.3	34.2	21.8	3.9	10.4

The colour of rose liqueurs was determined as *L*, a*, b*, c*, h** and *a/b* values, and was different both for rosa species and for variants.

The initial *L** values were from 60.1 do 77.7 for *R. rugosa* and *R. canina* liqueurs. Beverages made from *R. hybrida* were lighter (*L** = 76.8 – 82.9) and these from *R. spinosissima* were much darker (L = 11.4–13.5) due to anthocyanin content.

Liqueurs darkened with time of aging; The biggest changes in *R. canina* liqueurs were observed, especially in these, which were made according to variant V4.

Liqueurs showed a decrease in the *b** values and an increase in *a** values with aging time. Thus the *a/b* ratio, which describes sample browning, increased with storage time in liqueurs without anthocyanins. It is known that the polyphenolic components form brown complexes. Quinones produced by oxidation of polyphenols are reactive substances in some stages of the complex reactions leading to browning [Pokorny, 1980]. Total colour differences (ΔE), measured for every liqueur, significantly increased in time. The biggest changes of ΔE parameter (90.1) were in liqueurs from *R.canina* especially in one made according to variant V4 (fruit + sugar + ethanol 65% at the same time). In liqueurs from *R. hybrida* and *R. spinosissima* total colour difference parameter values were highest for samples made by variant V2 (fruit + sugar initially).

Maximum chroma *C**-values, which expressing colour brillance or purity were obtained for liqueurs from *R. canina*, *R. hybrida* and *R. rugosa* after 6 or 9 months of storage, and after three months for liqueurs from *R. spinosissima*. *C** increased over the period of 6 months in all samples.

Hue angle (h^o) gives a numerical estimate of the colour. The hue sequence on a CIE*Lab* diagram is defined with red-purple (0^o), yellow (90^o), bluish-green (180^o) and blue (270^o). Hue angle (h^o values) of liqueurs, except these from black rose, decreased during storage time towards orange-brown color. The largest decrease in the hue value was observed in *R. canina* liqueurs.

Colour of liqueurs is an important quality parameter. After aging our liqueurs had deep orange-brown color, typical (characteristic) for that kind of products (except samples made from *R. spinosissima*, which was dark-brown).

Liqueurs made from rosa fruit are rich source of phenolic compounds and showed high antioxidant activity, similar to those of cornelian cherry, myrtle and other liqueurs, industrial nectars [Vacca et al., 2003; Babis and Kucharska, 2004; Heinonen, 1998…] white wine [Sanchez Moreno et al., 1999]

Polyphenolic compounds extraction was somewhat better in variants V1 or V2, but fluctuations of total phenolic compounds were observed during aging. Antioxidant activity and polyphenol content was correlated (Table 5). Results was similar to observations Pijlac--Zegarac et al. [2009] in fruit juices.

Table 5

Correlation coefficients

	DPPH	ABTS	FRAP	Polyphenols
DPPH	1			
ABTS	0.51	1.00		
FRAP	0.40	0.65	1.00	
Polyphenols	0.37	0.81	0.88	1

In general, a little worse results of antioxidant activity in investigated samples were observed in liqueurs made according variants V3 and V4, and these from *R. rugosa* fruit.

The results of the study show that phenolic compounds of rose liqueurs undergo changes, especially in color, during storage. Fluctuations of phenolic content and antioxidant activity may be explained by tendency of polyphenols to undergo polymerisation and other reactions, resulting in reaction products that has higher or lower activity than initial compounds.

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6

CONTENT OF SOME BIOLOGICALLY ACTIVE COMPOUNDS IN THE EXTRACTS OF PRUNES AND THEIR ANTIOXIDANT ACTIVITIES

Introduction

Biologically active components of plants have recently attracted growing consumers' interests. It can be partially explained with their potential positive influence on human's health. It is known on a base of researches that a diet rich in fruits and vegetables helps in diminishing the risk of cardiovascular disease and some tumor types occurrence, and perhaps, it can protect against other diseases [Gibney et al., 2003]. Such protective effects of a diet have been greatly attributed to natural antioxidants present in fruits and vegetables, including vitamin C and β-carotene, as well as plant-origin polyphenols such as flavonoids or phenolic acids [Madhavi et al., 1995, Rao & Rao, 2007]. At the same time, an anxiety upon synthetic antioxidant application arose among consumers. There are *in vitro* studies that confirm the negative effects of β-carotene supplementation on smoker's health [Schiebier et al., 2001]. Therefore, it is important to make a public opinion realized the existence of natural products abundant in antioxidants – dried fruits. Unfortunately, dried fruits, despite of the fact that show strong pro-health properties and are characterized by high contents of vitamins and minerals, are not a common element of our everyday diet [Stahl & Sies, 2005].

The present study aimed at determining the contents of biologically active substances and testing the antioxidant capacity of extracts made of prunes. Contents of polyphenols, catechins, ascorbic acid, and carotenoids were determined. The antioxidant capacity was tested by means of using stable $DPPH^{\bullet}$ radicals, cation-radicals $ABTS^{\bullet+}$, and moreover, ability to chelate iron (II) ions was determined.

Material and methods

The experimental material consisted of prunes – three types purchased directly from different producers: in USA and Chile (Chile 1 and 2).

The chemical characteristics included determinations of total polyphenols contents by means of Folin-Ciocalteu's method (results were expressed as recalculated onto gallic acid) [Slinkard & Singleton, 1977, Singleton & Rossi, 1965], catechins applying vanillin method (results were expressed as recalculated onto (-)epicatechin) [Swain & Hillis, 1956], carotenoids [Sztangret et. al., 2001], and ascorbic acid contents using spectrofluorimetric method

[AOAC, 1990]. Determinations of total polyphenols, catechins, and anti-radical properties were performed using acetone extracts (70% v/v, material to extraction agent ratio 1:10), carotenoids in hexane extracts (material to extraction agent ratio 1:10), while vitamin C in metaphosphoric acid plus acetic acid mixture (1:2.5, m/v, material to extraction agent ratio 1:5). In addition, the ability to deactivate $DPPH^{\bullet}$ radicals was tested in chloroform solution [Saint-Cricq de Gaulejac, 1999].

The antioxidant capacity of analyzed extracts was determined by measuring their ability to neutralize the stable synthetic $DPPH^{\cdot}$ radicals. The method consists in adding the anti-radical substances into the methanol $DPPH^{\cdot}$ solution that shows the absorbance at λ=517 nm at its radical form. That value is decreased after adding anti-radical substance [Saint--Cricq de Gaulejac, 1999]. The anti-radical activity was then calculated from the difference of absorbance values for $DPPH^{\bullet}$ radicals solution before and after adding potential antioxidants [Lai et al., 2001, Saint-Cricq de Gaulejac, 1999]. $DPPH^{\bullet}$ is a synthetic radical that accepts one electron or hydrogen so that it could be converted in a stable DPPH molecule. The determination if often made to evaluate the antioxidant capacity due to the short analysis time and relatively high method's sensitivity [Durmaz & Alpaslan, 2007].

The anti-radical properties of extracts were also determined by examining their ability to deactivate cation-radicals $ABTS^{\bullet+}$ [Re et al., 1999]. The method consists in a direct generating the $ABTS^{\bullet+}$ resulting from ABTS oxidation by potassium hypersulfate. The antioxidant addition reduces $ABTS^{\bullet+}$ to ABTS, and the decrease of radical solution color intensity can be observed. The level of $ABTS^{\bullet+}$ reduction is measured spectrophotometrically at 734 nm wavelength.

Testing the components contained in the extracts referring to the ability to chelate iron (II) ions was carried out by adding the iron (II) chloride and ferrozine into the solutions. Absorbance of the color complex was measured after 10 minutes after ferrozine addition at 562 nm wavelength [Lai et al., 2001].

All determinations were made in five replications. Mean values and standard deviations were calculated with the help of Microsoft Office Excel 2003 software. The statistical analysis of the two-factorial experiment as well as correlation coefficients were calculated using STATGRAPHICS Plus 4.1 software.

Results and discussion

The study revealed that USA and Chile 1 prune samples contained similar levels of total polyphenols ranging from 760 to 786 mg/100 g DM. Content of polyphenols in sample Chile 2 was apparently lower amounting to about 500 mg/100 g DM (Table 1).

Table 1

The content of total polyphenols and catechins

Prunes	Total polyphenols [mg/100 g d..m.]	Catechins [mg/100 g d..m.]
USA	786.13 (±1.21) a	119.15 (±1.45) c
Chile1	759.72 (±1.43) a	291.74 (±1.10) b
Chile2	499.36 (±1.08) b	358.83 (±1.10) a

Explanatory notes:
Mean values followed by the same letter in a column don't differ significantly.

The variance analysis (ANOVA) for a single-factor experiment revealed statistically significant difference between mean values of polyphenols contents in particular samples of prunes (3 variability levels) at the significance level of 95%. Additionally made Tukey's test for the significance level $\alpha = 0.05$ allowed for grouping the samples in uniform groups, in which no statistically significant differences occurred. Such analysis indicated that no uniform group can be distinguished among studied prune samples.

According to literature data, the total polyphenols contents in prunes oscillates in wide range from 600 mg to 1500/100 g DM [Cieślik et al., 2006, Hooshmand & Arjmandi, 2009]. It should be mentioned that many factors such as the extraction type or material particle diameter affect the amount of determined polyphenols; also the extraction times can be different (from 1 minute to 24 hours) [Naczk & Shahidi, 2004]. Excessively long extraction time increases the risk for polyphenols oxidation, until reducing reagents are added to the system. Moreover, researchers also pay attention to the fact that the ratio of analyzed material weight to the volume of applied extracting solvent has detrimental effect of the determination results. In the case of their studies, the enhancing the ratio from 1:5 to 1:10 (w/v) caused the increase of condensed tannins extraction efficiency from 257.3 mg to 321 mg/100 g DM when 70% (v/v) acetone was used as extraction agent. Some author also proved that polyphenols contents determination results in dried beans are also strongly affected by the particle size of studied material. Dragovic-Uzelac et al. [2005] recorded that quantitative differences of total polyphenols contents may occur depending on the fruit variety, its maturity, growing conditions, and storage.

The total catechins content in examined extracts from prunes varied. The most catechins were found in sample Chile 2 (358.83 mg/100 g DM), while the least in sample USA USA (119.15 mg/100 g DM). The variance analysis (ANOVA) revealed statistically significant differences between mean values for studied samples at the significance level of α=0.05. Applied Tukey's test did not allow for distinguishing any uniform group among analyzed ones.

The reasons for such great differentiation of catechins contents may be the same as in the case of polyphenols. The fruit nutritional value depended mainly on the species and cultivation localization [Tarhan, 2007].

Among examined prunes, the highest vitamin C content was attributed to sample Chile 2 – 5.86 mg%, whereas the lowest – sample Chile 1 – 3.34 mg% (Table 2).

Variance analysis (ANOVA) indicated statistically significant differences between mean values for all sample groups at the significance level of $\alpha = 0.05$. The Tukey's test did not allow for distinguishing any uniform group among analyzed ones.

Table 2

The content of vitamin C and total carotenoids

Prunes	Vitamin C [mg/100 g d.m.]	Total carotenoids [mg/100 g d.m.]
USA	3.84 (±0,59)	0.82 (±0,76)
Chile1	3.34 (±0,43)	0.90 (±0,87)
Chile2	5.86 (±0,67)	0.92 (±0,45)

The literature references indicated that average ascorbic acid contents in prunes should be about 5.6 mg% [Kunahowicz et al., 2005]. Among here examined samples, only one prune sample contained similar level of vitamin C. Other samples contained lower vitamin C concentrations. Levels of vitamins and minerals may considerably vary in fruits depending on the cultivation site and soil composition. Furthermore, it is well known that fruit ripeness level also significantly affects the nutritional quality of fruits and vegetables [Munzuroglu, 2003]. More ripened (or over-ripened) fruits are characterized by lower vitamin C content than those at technological maturity stage [Karatas & Kamisli, 2007].

The carotenoids contents ranged at relatively low, although uniform, level for all examined extracts (from 0.8 to 0.9 mg/100 g DM). From literature data, it follows that depending on the fruit variety, carotenoid concentrations can oscillate from 0.3 to 0.8 mg/100 g DM. However, the fact that final results always depend on the extraction technique and applied method, should be on mind [Chun et al., 2006, Kim et al., 2007]. The variance analysis (ANOVA) for the results of the experiment carried out using prunes at the significance level of $\alpha = 0.05$ did not indicated statistically significant differences between analyzed mean values (p-Value 0,44). Moreover, Tukey's test (for $\alpha = 0.01$) allowed for qualifying all samples to the same homogenous group.

The determination of extract's abilities to deactivate the stable DPPH$^{\bullet}$ radicals was performed in two extracting systems:

- acetone;
- chloroform.

The extraction conditions were in both cases analogous, only the extracting reagents were different. For acetone, mainly polyphenols were extracted, while in the case of chloroform, lypophilic substances such as carotenoids, were extracted.

All examined prune acetone extracts showed the ability to deactivate DPPH$^{\bullet}$ radicals at similar level of 93–94% (Fig. 1).

On a base of literature data, it can be suggested that here studied antioxidant capacity greatly depended on the method type applied for the extraction and that observed antioxidant capacity not ways correlated with total polyphenols contents in plant extracts [Cevallos-Casalas et al., 2006].

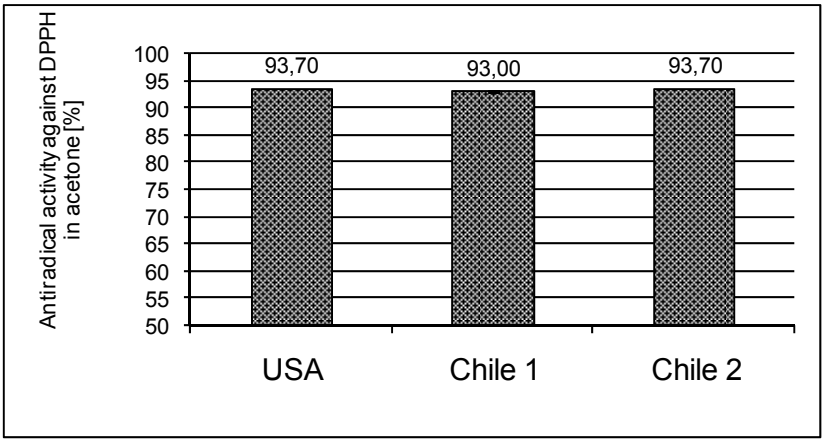

Fig. 1. Anti-radical activity against DPPH$^{\bullet}$ in acetone [%]

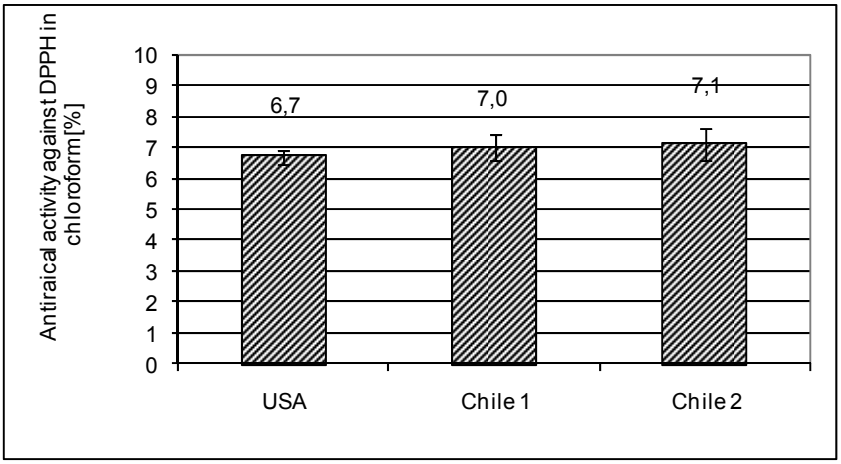

Fig. 2. Anti-radical activity against DPPH$^{\bullet}$ in chloroform [%]

Other authors reported that probably condensed tannins are the substances mostly responsible for the effect of stable DPPH$^{\bullet}$ radicals sweeping off [Falleh et al., 2008]. In present study, no condensed tannins contents were determined, thus it is impossible to direct verifying the correlation between these compounds concentration and ability to sweep the DPPH$^{\bullet}$ radicals. The regression analysis at 90% confidence interval, confirmed the existence of a correlation between total polyphenols contents and the ability to inhibit DPPH$^{\bullet}$ radicals by prune extracts.

Chloroform extracts were characterized by much poorer ability to deactivate stable DPPH$^{\bullet}$ radicals than acetone ones. Extracts made of prunes neutralized free radicals at similar level (about 7) (Fig. 2). The chloroform extracts contained mainly carotenoids (although polyphenols are the strongest and the most active antioxidants), hence it can be supposed that it is the reason of lower ability of these extracts to deactivate radicals than those made of acetone that contain mainly polyphenols [Gardner et al., 2000]. The regression analysis revealed positive correlation between total carotenoids contents and the ability to sweep off the DPPH$^{\bullet}$ radicals at the significance level of $\alpha = 0.01$ (p-Value = 0.002) for analyzed prune extracts samples.

The ability of examined extracts to deactivate cation-radicals ABTS$^{\bullet+}$ was also tested in the study. Determinations were carried out according to modified method described by Re et al. [1999]. The surplus of modified method over the traditional one consists in the fact that ABTS$^{\bullet+}$ radicals are formed in a system directly, with no participation of mediated radicals, and anti-radical compound is added into the solution just after creating the cation-radicals. Thus, the risk of errors associated with the reaction of examined compound with mediated radicals, is eliminated. It was found that all studied extracts showed the ability to inactivate synthetic cation-radicals ABTS$^{\bullet+}$ (Fig. 3). Among tested prunes, samples designated as USA and Chile 1 were characterized by the ability to sweep off the cation-radicals at similar levels (48.8% and 48.5%, respectively), while sample Chile 2 deactivated these radicals at 30%.

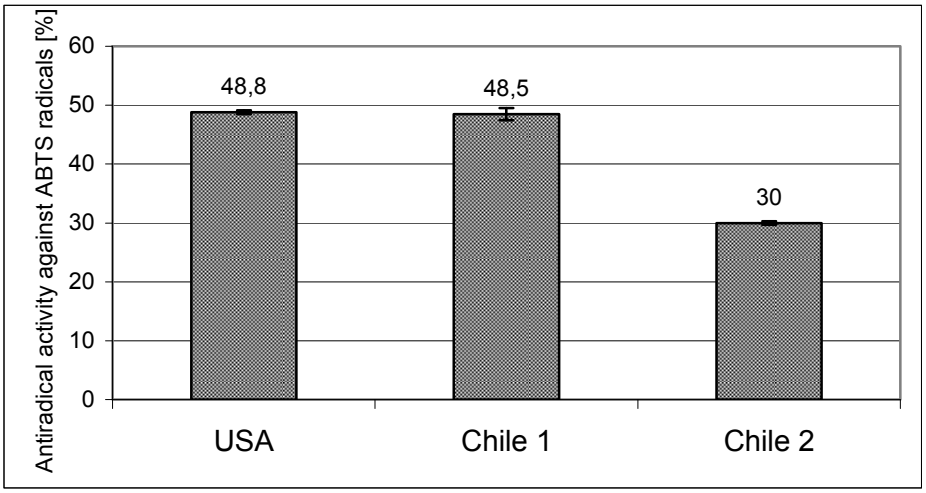

Fig. 3. Anti-radical activity against radicals ABTS [%]

Other authors reported that polyphenols contents was strongly correlated with the ability of ABTS radicals deactivation [Falleh et al., 2008, Panalla et al., 2001]. Here performed analysis of the associations between total polyphenols concentration vs. $ABTS^{\bullet+}$ cation-radicals sweeping-off ability indicated a strong positive correlation for prune extracts (p-Value=0.002) at 99% significance level.

It should be also mentioned that due to various modifications of the method for anti-radical ability against $ABTS^{\bullet+}$ determination, it is difficult to compare results in different researches.

The present study dealt with the determination of the chelating the iron (II) ions by acetone extracts. The results indicated that all analyzed extracts showed such abilities (Fig. 4).

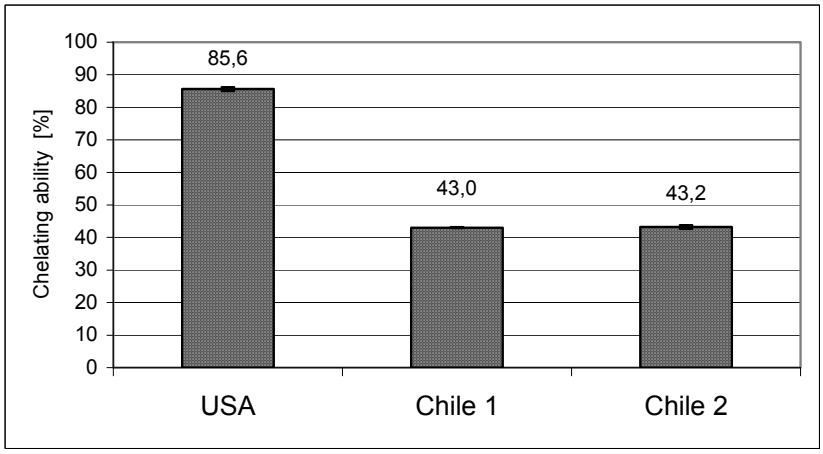

Fig. 4. Chelating iron ion ability [%]

Among prune extracts, sample from USA chelated iron (II) ions in 85%, while both samples originating from Chile were characterized by half of that ability. According to some authors, mainly polyphenols have the property to bind transitional metals ions [Sanchez-Moreno et al., 1998].

The regression analysis indicated the positive correlation at α=0.05 significance level (p-Value = 0.027) between total polyphenols contents vs. ability to chelate iron (II) ions in prune extracts. Such dependence was also observed in other authors' works [Gardner et al., 2000].

Conclusions

1. Polyphenols dominate among studied prune samples in their biological activity. Catechins contents in analyzed samples were recorded at varied, yet significant levels.

2. All examined extracts showed the ability to sweep off the stable $DPPH^{\bullet}$ radicals and cation-radicals $ABTS^{\bullet+}$. All extracts also indicated the ability to chelate iron (II) ions.

3. Prunes are a valuable source of biologically active substances (polyphenols, ascorbic acid, carotenoids), hence they should be included in our everyday diet.

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7

ATTEMPTS TO INCREASING OF OXIDATIVE STABILITY OF SUNFLOWERSEED AND RAPESEED COLD PRESSED AND FULLY REFINED OILS BY USING GREEN TEA EXTRACT

Introduction

Lipid oxidation is most important factor affecting flavour quality and shelf life of vegetable oils. The oxidative stability may be influenced by many factors, such as fatty acid composition, the presence of natural antioxidants and pro-oxidants and additionally by the storage conditions, such as: temperature, presence of light and oxygen [Szukalska 1999, Drozdowski 2007]. The products of lipid oxidation may be dangerous for human health and are associated with aging, membrane damage, heart diseases and cancer [Kubow 1990; Ziemlański, Budzyńska-Topolowska 1991; Scrimgeour 2005].

Nowadays various synthetic and natural antioxidants are used for prevention of lipid oxidation. Because of possible some negative side effects of the commonly used synthetic antioxidants (BHA and BHT) there is an increasing interest to replace these antioxidants by natural ones. The use of natural extracts is now a strong trend in both research and industrial applications. Various medical plants and herbs, spices [Madsen & Bertelsen 1995; Marinova & Yanishlieva 1997; Yanishlieva et al., 2006], tea [Chen, Ho 1995; Chen, Chan 1996; Wang et al., 2000; Gramza, Korczak 2005], grains, fruits and vegetables are the main sources of natural antioxidants [Madsen & Bertelsen 1995, Yanishleva & Marinova, 2001].

In recent years tea has attracted significant attention because of reported health benefits because of the antioxidant activity. The flavonoids are mainly responsible for this action. Green tea leaves contain 30–42% poliphenols, on a dry weight [Wang et al., 2000; Wierzejska 2004]. Catechins are the predominant group of green tea poliphenols and are comprised of (-)epicatechin, (-)epicatechin gallate, (-)epigallocatechin, (-)epigallocatechin gallate, (+)catechin and (+)gallocatechin [Chen, Chan 1996, Wang et al., 2000, Ostrowska 2001, Gramza, Korczak 2005]. Catechins and other flavonoids have been recognized as efficient antioxidants for scavenging oxygen radicals and chelating metal ions [Chen, Ho 1995].

Recently, interest in cold pressed oils also has grown, because they are seen as more healthy then fully refined oils [De Panfilis et al., 1998, Koski et al., 2002]. They contain usually more natural beneficial ingredients – antioxidants, such as tocopherols, sterols, carotenoids, and phospholipids then fully refined oils [Prior et al., 1991,a,b; Koski et al., 2003]. However cold pressed oils from seeds are less stable then fully refined oils [Kristott 2000, Wroniak et al., 2006].

In order to increase the oxidative stability of cold pressed rapeseed and sunflowerseed oils and for comparison fully refined oils, natural antioxidant – extract of green tea was chosen. The aim of the study was to compare the influence of green tea extract on oxidative stability of cold pressed oils and fully refined oils.

Materials and Methods

A commercially available samples of oils: rapeseed and sunflowerseed cold pressed oils and fully refined oils in 1l PET bottles from local plants and extract of green tea - powder 20% of catechins – Guardian Green Tea Extract OS, from Danisco and BHT (butylated hydroxytoluene) from Merck were used. In tests both antioxidants were used in following doses: 0,02, 0,04, 0,06, 0,1, 0,2%.

The quality of cold pressed oils was estimated with: peroxide value according to PN-ISO 3960:1996, fatty acid composition according to PN-ISO 5509:2001, PN-EN ISO 5508:1996. For GC analysis a HEWLETT-PACKARD 5890 Series II chromatograph equipped with a flame-ionization detector (FID) and capillary column BPX 70 (60 m x 0,25 mm) was used. The temperature program was 140–210°C. The injection and detector temperatures were 210 and 250°C, respectively. Helium was used as carrier gas.

Oxidative stability as induction time was determined by Rancimat test at 120ºC (typ 679 Metrohm), according to PN-ISO 6886:1997. The effectiveness of antioxidant was expressed by calculating a stabilization (protection) factor (F):

$$F = (IPa – IP)/IP,$$

where:
IPa – induction time with antioxidant,
IP – induction time in the absence of the extract in oil.

Additionally acid value are determined according to PN-ISO 660:1998.

The obtained results were analyzed statistically with the computer program STATGRAPHICS PLUS 4.1. Means and standard deviations (SD) were calculated. Variant analysis was used to check the significance of statistical differences between the means at $p \leq 0.05$.

Results and discussion

Quality and stability of examined oils. Very important quality factor especially of pressed oils are acid and peroxide values (Table 1). Acid value of pressed oils were 1,79 in rapeseed oil and 2,08 mg KOH/g in sunflowerseed oil (standard maximum 4). Peroxide value of pressed oils were 1,86 in rapeseed oil and 8,43 meq O_2/kg in sunflowerseed oil (standard maximum 15) [Codex Alimentarius, 2001]. Of course both, peroxide and acid values were much lower in fully refined oils. Acid value were 0,21 in rapeseed oil and 0,36 mgKOH/g in sunflowerseed and peroxide value were 0,31 and 0,89 meq/kg, respectively. At the stage of deodorization free fatty acids were removed as well as the products of fat auto-oxidation which was reflected in the minimum values of acid and peroxide value.

Taking into consideration the international legislation, according to obtained results of acid value, peroxide value, the analysed samples had proper quality for cold pressed oils and fully refined oils, respectively.

Table 1

The quality parameters and fatty acids composition of examined cold pressed and fully refined oils

Oils	cold pressed rapeseed	cold pressed sunflowerseed	fully refined rapeseed	fully refined sunflowerseed
Acid value [mg KOH/g]	1,79±0,01[a]	2,08±0,01[b]	0,21±0,01[c]	0,36±0,01[d]
Peroxide value [meq O_2/kg]	1,86±0,02[a]	8,43±0,03[b]	0,31±0,01[c]	0,89±0,01[d]
Rancimat 120°C induction time [h]	3,60±0,11[a]	1,65±0,13[b]	5,15±0,10[c]	2,65±0,07[d]
Fatty acids [%]				
14:0	0,1±0,0	0,1±0,0	0,1±0,0	0,1±0,0
16:0	4,4±0,2	6,1±0,1	4,6±0,1	6,3±0,2
16:1	0,2±0,0	0,2±0,0	0,4±0,1	0,1±0,0
17:0	0,1±0,0	-	0,1±0,0	-
17:1	0,1±0,0	-	0,1±0,0	-
18:0	1,7±0,1	3,7±0,2	2,0±0,1	3,1±0,1
18:1 cis	61,2±0,3	23,7±0,2	59,2±0,1	24,6±0,2
18:2 trans	-	-	0,1±0,0	0,2±0,0
18:2 cc	19,6±0,2	63,6±0,2	18,9±0,1	62,0±0,3
18:3 trans	-	-	0,2±0,0	-
18:3 ccc	6,8±0,1	0,1±0,0	8,2±0,2	1,1±0,1
20:0	0,7±0,1	0,2±0,0	0,6±0,1	0,2±0,0
20:1	2,8±0,1	0,5±0,1	2,6±0,1	0,3±0,1
20:2	0,1±0,0	0,1±0,0	0,1±0,0	0,1±0,0
22:0	0,5±0,1	0,7±0,1	0,4±0,1	0,7±0,1
22:1	0,9±0,1	0,1±0,0	1,6±0,1	0,2±0,0
24:0	0,2±0,0	0,1±0,0	0,1±0,0	0,2±0,0
24:1	0,1±0,0	0,1±0,0	0,2±0,0	0,1±0,0

* – values marked with the same letter in a row are not significantly different at $p \leq 0.05$,
all values are mean of three replicates ± standard deviation.

Sunflowerseed oils showed about 50% lower oxidative stability than rapeseed oils, induction time was 1,65 and 2,65h and 3,60 and 5,05 h, respectively for cold pressed and fully refined oils (Table 1). These differences are statistically significant. However, the initial degree of oxidation of both oils should be taken under consideration, which is significantly higher in the case of the cold pressed oil (Table 1). These results were similar to those obtained in other investigations [Wroniak et al., 2006].

For all the examined oils the composition of fatty acids was typical [Codex Alimentarius, 2001]. The method of oil production (cold pressed and refined) did not change its composition of particular fatty acids (Table 1). However cold pressed oils did not contain trans isomers of fatty acids. They are formed mainly during deodorization when a very high temperature up to 240°C is applied. The characteristic feature of rapeseed oils is the presence of the erucic acid (C22:1), from 0,9 to 1,6%. The fatty acids composition of the oils

can partially explain differences in oxidative stability in Rancimat test. Sunflowerseed oils had higher content of polyunsaturated fatty acid then rapeseed oil (especially C18:2 content) and showed about 50% lower induction time in Rancimat in both – cold pressed and fully refined oils (Table 1).

Influence of green tea extract on oils stability. Table 2 showed the effects of added antioxidants (green tea extract and BHT) on induction time in Rancimat test, and Figures 1 and 2 show results expressed as the stabilization factors (F). The antioxidant activity of added extract was dependent on used doses 0,02, 0,04, 0,06, 0,1, 0,2%. Substantial improvement of oxidative stability was observed in oils in the case of both used antioxidants. The higher doses gave the longer induction time in Rancimat test in all tested oils.

Table 2

The induction time in Rancimat test for antioxidants in rapeseed and sunflowerseed cold pressed oils and fully refined oils

Oils	Induction time [h]							
	cold pressed rapeseed		cold pressed sunflowerseed		fully refined rapeseed		fully refined sunflowerseed	
Dose [%]	green tea extract	BHT	green tea extract	BHT	green tea extract	BHT	green tea extract	BHT
0	3,60[a]		1,65[a]		5,15 [a]		2,58 [a]	
0,02	3,94 [ab]	3,64 [a]	1,66 [a]	1,81 [ab]	6,25 [b]	5,30 [b]	3,19 [b]	3,04 [b]
0,04	4,02 [b]	3,69 [a]	1,83 [ab]	1,85 [ab]	7,42 [c]	5,42 [bc]	3,76 [c]	3,05 [b]
0,06	4,19 [bc]	3,82 [b]	1,94 [abc]	2,08 [bc]	8,28 [d]	5,48 [bc]	4,17 [c]	3,13 [b]
0,10	4,50 [c]	4,40 [c]	2,10 [bc]	2,24 [c]	8,72 [e]	5,62 [c]	4,36 [d]	3,55 [cd]
0,20	5,10 [d]	4,90 [d]	2,29 [c]	2,90 [d]	9,52 [f]	5,84 [d]	4,86 [e]	3,64 [d]

* – values marked with the same letter in a column are not significantly different at $p \leq 0.05$.

Taking into account the kind of tested oils was stated, that the better efficiency of added green tea extract was demonstrated in fully refined then in cold pressed oils. Any increase in dose resulted in a significant increase of the oxidative stability of tested refined oils. It was found that applied a dose 0,02% resulted in marked increase in the stability of all tested oils. In fully refined sunflowerseed oil, this increase was up to 23,6%, for rapeseed oil to 21% (Table 2). The highest stabilization factor was observed in sunflowerseed oil for maximum dose 0,2% (F = 0,88). This antioxidant extended the induction time in sunflowerseed oil from 2,58 to 4,86 h (about 88%) and in rapeseed oil from 5,15 h to 9,52 h (about 85%). It was stated that green tea extract in a similar degree protected rapeseed and sunflowerseed fully refined oils at all doses.

In cold pressed oils the act of green tea extract was less effective. The addition of this extract in dose 0,02% in cold pressed oils both: rapeseed and sunflowerseed showed little antioxidative effect toward the oils without antioxidants. The induction time extended only about 42%, from 3,60 to 5,10 h in rapeseed oil and similarly about 39%, from 1,65 to 2,29 h in sunflowerseed oil, for maximum added dose 0,2% (Table 2). For rapeseed oil values of protection factors (F) ranged from 0,10 to 0,42 while results obtained for sunflowerseed oil were from 0,06 to 0,39 (Fig. 1). It may be considered that the effectiveness of tea extract did not differ in both tested cold pressed oils (rapeseed and sunflowerseed) similarly like in fully refined.

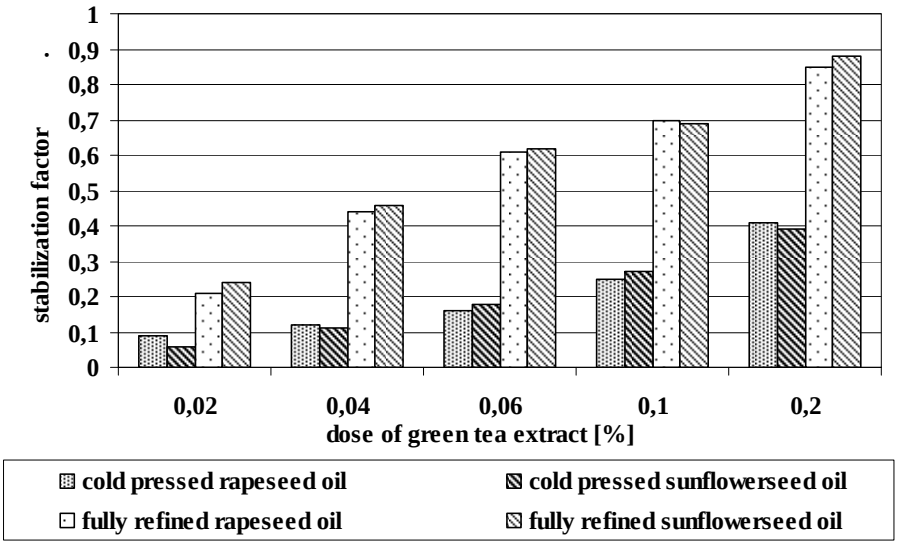

Fig. 1. Effects of green tea extract on stabilization factor of rapeseed and sunflower cold pressed oils and fully refined oils

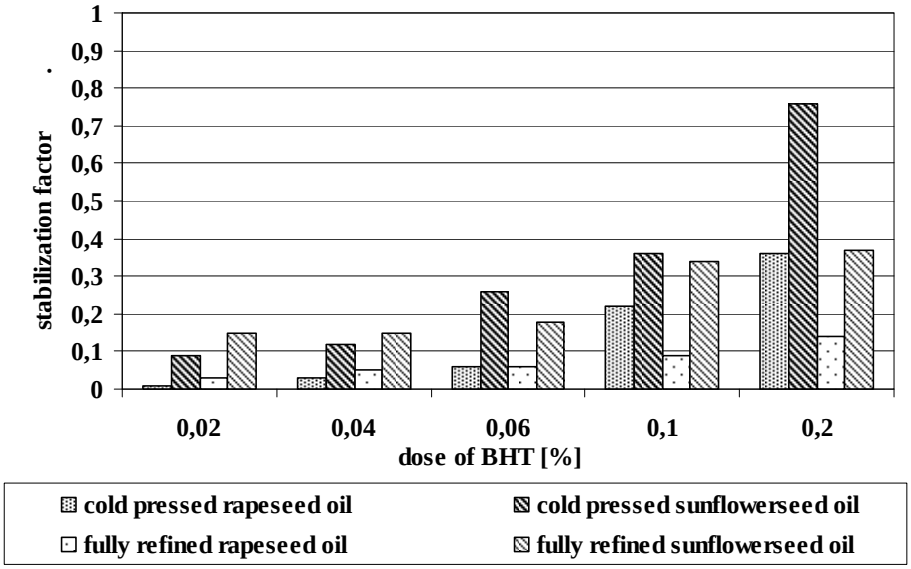

Fig. 2. Effects of BHT on stabilization factor of rapeseed and sunflower cold pressed oils and fully refined oils

Better results obtained Chu and Hsu [1999] using catechin alone and composites with other antioxidants to stabilize peanut oil and showed significant increasing of oil stability as compared with control oil. They stated that addition of catechin in quantities 0,1% caused in increase induction time in OSI (Oxidation Stability Index) of 100% (9,44 h), and in 0,2% of 290% (18,58 h) to control (4,77h). Also Yanishlieva, Marinova [2001] investigated the flow of green tea extract for the length of the induction period of rapeseed oil in the accelerated oxidation test (temp. 100˚C) and stated that addition in quantities of 0,02% to oil has extended the induction time.

However, it should be remembered that cold-pressed (unrefined) oils may contain a number of associated compounds prooxidants (metal ions, chlorophylls, free fatty acids, the products of oxidation) and on the other hand natural antioxidants (tocopherols, sterols, phospholipids, carotenoids) which may interfere with effectiveness of the added antioxidants.

Influence of BHT on oils stability. The results showed that the green tea extract was more effective then BHT in examined refined oils (Table 2). The lowest stabilization factor was stated by samples with added BHT in fully refined oils, especially in rapeseed oil (F = 0,14 for maximum dose) (Fig. 2). In some studies were stated less efficiency of BHT influence on the oxidative stability of oils than some natural antioxidants used in the same concentrations. This difference could be explained by higher volatility of BHT at high temperature of 120°C used with air flow in Rancimat test [Nogala-Kałucka et al., 2005]. Chen, Chan [1996] concluded that thermal loss of the catechins was significantly less compared to that of BHT in canola oil heated at 95°C. Faster thermal loss of BHT was attributed to volatilization and stream distillation due to high temperature. They stated that green tea catechins were more protective then BHT against lipid oxidation in canola oil in high temperature and may replace BHT as antioxidants in processed food. In other investigations, the extracts of green tea up 0,02% reduced the formation of peroxides more effectively, then α-tocopherol, BHA and BHT in marine oils [Wanasundara & Shahidi, 1998].

A different situation was observed in the case of cold pressed oils (Fig. 2). BHT was very effective and better than green tea extract in the increase of oxidative stability especially of cold pressed sunflowerseed oil for all examined doses. The best results were obtained using very high dose 0,2% of BHT. The induction time of sunflowerseed oil was increase from 1,65 to 2,9 h (about 76%) and of rapeseed oil from 3,60 to 4,9 h (36%) at this dose (Table 2). Values of stabilization factor for cold pressed rapeseed oil for BHT were significantly lower then in sunflowerseed oil (Fig. 1, 2).

On the basis of induction time in Rancimat test and calculated stabilization factor it was stated that the most effective in a case of fully refined oils was green tea extract, while in cold pressed oils BHT (especially in sunflowerseed oil). Native antioxidants in a cold pressed oils may significantly affect the stabilizing effect from antioxidant addition.

Conclusions

1. The oxidative stability of cold pressed oils was lower then refined oils: in the case of rapeseed oils induction time was 3,60 and 5,15 h, respectively and in the case of sunflowerseed oil induction time was 1,65 and 2,58 h, respectively.

2. Rapeseed oils, both cold pressed and fully refined, are about 50% more stable than sunflowerseed oils.

3. The highest efficiency was demonstrated by extract of green tea in fully refined oils: sunflowerseed oil (F = 0,88) and rapeseed oil (F = 0,85), while much lower in cold pressed oils: rapeseed (F = 0,42) and sunflowerseed (F = 0,39) for maximum dose (0,2%).

4. Generally lower efficiency of BHA in comparison with green tea was observed at all doses in all oils, except of cold pressed sunflowerseed oil.

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8

CHANGES IN ACTIVITY OF PROTEOLYTIC AND AMYLOLYTIC ENZYMES INDUCED DURING GERMINATION OF PLANTS IN $FeSO_4$ SOLUTIONS

Introduction

Supplementation of food with substances expressed in plants during germination is very promising form of diet fortification with elements supplied in inadequate amounts, such as microelements, vitamins, antioxidants and dietary fiber [Bau et al., 1997; Martin-Cabrejas et al., 2003; Zieliński et al., 2005a; Zieliński et al. 2005 b]. Presented experiments were conducted to obtain sprouts biofortified in iron and to check an activity of hydrolytic enzymes significant to industrial food preparations.

Fortification of plants with iron is one of the strategies of anemia prevention, because its deficiency in human diet is observed in up to 30% of the population [Theil, 2004]. Plants were germinated under high abiotic stress conditions, caused by high concentration of ferrous sulfate in the culture medium. During plant growth a high accumulation of iron was observed [data in press, "Żywienie Człowieka i Metabolizm", 2009]. Growing plant protects its cells against formation of free radicals, introducing the iron *inter alia* into ferritn [Deák et al., 1999]. In consequence, a high bioavailability of iron in a 'safe form' from the sprouts is expected.

An addition of processed sprouts biofortified with iron can be an excellent choice for improving the nutritive value of various food products. However, iron concentration in the culture medium induces also some others defensive mechanisms in plants, such as changes in the expression of other specific proteins, not only ferritin. These changes are observed in the expression of heat shock proteins as well as hydrolytic enzyme groups, protein inhibiting amylase proteases and lectins [Leah and Mundy, 1989]. The capacity of plant adaptation to unfavorable environmental factors is not completely clarified [Domash et al., 2008].

Hydrolysis is the most universal process in nature. Hydrolytic enzymes become active during germination, on the one hand causing decomposition of storage substances of the endosperm, on the other hand preparing amino acids, low molecular weight peptides and monosaccharides for cell growth. Under stress condition, a disturbance in the level of hydrolytic enzymes activity causes severe problems in plant development.

Data on the dependence of hydrolytic enzymes activity under stress conditions is poorly available [Domash et al., 2008]. The level of hydrolytic enzymes in sprouts biofortified with iron may be an interesting issue, because these enzymes are of great importance in food technology, especially in cereal processing.

From the point of view of technological properties of raw materials in food industry, the most important enzymes are amylases and proteases. In baking technologies hydrolysis of starch should be controlled because it increases viscosity and accelerates fermentation processes, this influencing crumb structure and elasticity as well as loaf volume. Protein decomposition is connected to changes in water absorption of gluten and its rheological properties [Dojczew et al., 2004; Miś, 2002]. A higher activity of these enzymes increases the presence of simple peptides, amino acids and monosaccharides, which also facilitate growth of undesirable microflora.

Changes in activity of these enzymes as a result of stress condition during the plant growth were analysed in sprouted soy bean, alfalfa and wheat.

Materials and methods

Materials

Experiments were carried out on:

- soy bean seeds (*Glycine max*, cv. Naviko), obtained from Department of Genetics and Plant Breeding Poznan University of Life Sciences, Poland,
- alfalfa seeds (*Medicago sativa* L., cv. Tula) obtained from Poznan Plant Breeders Ltd. In Tulce, Poland,
- wheat grain (*Triticum aestivum* L., cv. Bombona) obtained from Danko Plant Breeders Ltd., Choryń in Poland.

All chemicals used in presented experiments were analytical grade and purchased from POCh (Poland) and Sigma-Aldrich.

Methods

Sprouts preparation. Seeds and grains were soaked in 70% ethanol solution for 15 minutes at room temperature for disinfection. After washing out of ethanol from the seeds and grains with tap and distilled water, dry grain and seeds were soaked for successive 12 hours in $FeSO_4$ solutions (0–25 mM $FeSO_4$). Afterwards samples were cultured in special germination dishes for 7 days at room temperature. They were watered every day with fresh $FeSO_4$ solution with respective concentrations. Finally obtained sprouts were dried in a stream of warm circulating air to 8–10% of moisture content. Samples of dried sprouts were milled and stored in the powder form in tightly sealed containers at room temperature. Prepared sprouts obtained from three replications of culture were mixed together.

Extract preparations. Extracts were prepared from processed sprouts in one-step and one-hour extraction (1:10 m/v) with 0.1 M acetic buffer, pH 5.3. Obtained supernatants were frozen and stored at -20^{o}C until the next day.

Protein determination in extracts. The extractable protein content was determined at 750 nm according to Lowry [1951].

Amylolytic activity determination. Specific amylolytic activity was determined according to Bernfeld [1955]. Blank samples were prepared from thermally denaturated extracts. The unit of amylolitic activity (JAA) is defined in micromoles of maltose ($E^{\mu M} = 0.558$), which is formed from starch during 1 min of hydrolysis performed by 1 mg protein extracted form prepared sprouts.

Proteolytic activity determination. Specific proteolytic activity was analyzed by a modified Fritz method. Samples were buffered by 0.1 M Tris-HCl, pH 7.8, 1% substrate solution was prepared in DMSO. Temperature of incubation was 37°C. Blank samples were prepared from appropriate extracts after protein precipitation. The precipitation was carried out by TCA method. The unit of proteolytic activity is defined in micromoles p-Nitroaniline ($E^{\mu M}$ = 0.0088) prepared from hydrolysis of 1% N_{α}-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride during 1 minute by 1 mg protein extracted form prepared sprouts.

Statistical analysis. Statistical analyses were performed using Statistica 8.0. All the data were expressed as means ± standard deviation and were subjected to analysis of variance ($p<0.05$). Every experiment was repeated three times.

Results and discussion

Germination under presented conditions induces oxidation stress in plants. The presence of heavy metals, particularly iron, in the environment disturbs cell metabolism, because many of those metals have a high affinity to sulfur-containing ligands and react with the -SH groups of enzymes [Domash et al., 2008].

In experiments presented before it was recognized, that high concentrations of iron during germination induce significant changes in biomass appearance. Sprouts are much shorter and, as well as seeds/grain, are discoloured brownish red or even black. The bigger the $FeSO_4$ concentration is, the more evident changes are observed [Zielińska-Dawidziak and Twardowski, 2008]. Tested plants i.e. soy bean and alfalfa seeds and wheat germs, also have different levels of tolerance to iron sulfate concentration. Tolerance of the legume plants is higher, species-specific and it can be related to ferritin expression [Zielińska-Dawidziak and Twardowski, 2008]. The difference in the tolerance (i.e. 0-25 mM for legumes and 0-5 mM for wheat) resulted in different concentrations used in time of plant culturing, Higher concentration of $FeSO_4$ in the culture medium strongly inhibited germination.

The determination of amylolytic activity was based on reducing sugar content. As it was expected, presented results showed that a higher activity of amylases was observed in sprouts cultured in distilled water (0 mM $FeSO_4$) than in raw seeds and germs. For wheat-germs and alfalfa seeds, the effect of increasing iron concentration in the growing medium on the inhibition of amylase activity or expression was observed (Tab.1–2). However, the tendency was found only in a specific range of $FeSO_4$ concentrations, i.e. 1–4 mM, for wheat germination. Very interesting results were obtained for the iron concentration which is recognized as significantly limiting the sprouting of wheat (>5 mM $FeSO_4$) and even possibly causing plant death. In that culture concentration for wheat grain the amylases activity was higher than for the lower concentration of $FeSO_4$. It may result from two different processes: the first one may be the inhibition of expression of amylase inhibitors, the second is a special α-amylase secretion as the result of high abiotic stress. This special α-amylase act even after cell death induced by abiotic or biotic stress [Doyle et al., 2007].

In germinated soy bean the results are different: the activity of amylases increased up to the applied level of 20 mM $FeSO_4$ in the culture (Tab 3). The results suggested a higher tolerance or even stimulation of activity of tested soy bean amylolytic enzymes to Fe^{2+} concentration in the culture medium. Further increasing of iron concentration up to 25 mM in growth medium decreases the specific activity of amylases, as it was observed for wheat-germs and alfalfa sprouts.

Table 1

Amylolytic and proteolytic activity of wheat grain and wheat-germs cultured in $FeSO_4$ solutions

Sample	Concentration of $FeSO_4$ in culturing medium [mM]	Extractable protein [mg/100 g dry mater]	Specific amylolytic activity [JAA]	Specific proteolytic activity [JAP]
non-germinated wheat grain	–	1.96±0.23 a	11.05±0,37 *d*	3.95±0,58 *e*
germinated wheat	0	2.79±0.47 *b*	12.65±0,13 *e*	2.67±0.13 *c*
	1	3.11±0.39 *c*	9.15±0,07 *c*	1.91±0.32 *a*
	2	3.38±0.39 *e*	7.70±0,06 *b*	1.99±0.36 *a*
	3	3.21±0.42 *d*	7.01±0,03 *a*	3.84±0.03 *d*
	4	3.18±0.34 *d*	6.99±0,02 *a*	2.36±0.26 *b,c*
	5	3.23±0.39 *d*	9.56±0,04 *c*	2.21±0.33 *b*

Values (mean ± standard deviation) with different index letters are statistically significantly different (P<0.05)

Table 2

Amylolytic and proteolytic activity of alfalfa seeds and sprouts cultured in $FeSO_4$ solutions

Sample	Concentration of $FeSO_4$ in culturing medium [mM]	Extractable protein [g/100 g dry mater]	Specific amylolytic activity [JAA]	Specific proteolytic activity [JAP]
non-germinated alfalfa seeds	–	28.35±0.23 *e*	0.01±0.00 *b*	0.80±0.02 *e*
Germinated alfalfa	0	30.29±0.46 *f*	0.02±0.00 *c*	1.07±0.08 *f*
	5	28.15±0.23 *e*	0.02±0.00 *c*	0.69±0.02 *d*
	10	27.68±0.39 *d*	0.02±0.00 *c*	0.72±0.00 *d,e*
	15	26.32±0.34 *c*	0.02±0.00 *c*	0.48±0.01 *c*
	20	24.01±0.34 *b*	0.01±0.00 *b*	0.24±0.02 *b*
	25	23.09±0.42 *a*	0.00±0.00 *a*	0.10±0.00 *a*

Values (mean ± standard deviation) with different index letters are statistically significantly different (P<0.05)

Table 3

Amylolytic and proteolytic activity of soy-bean seeds and soy bean sprouts cultured in $FeSO_4$ solutions

Sample	Concentration of $FeSO_4$ in culturing medium [mM]	Extractable protein [g/100 g dry mater]	Specific amylolytic activity [JAA]	Specific proteolytic activity [JAP]
non-germinated soy bean seeds	–	32.10±0.46 *e*	0.57±0.00 *a*	0.68±0.1 *a*
germinated soy bean	0	29.60±0.23 *d*	1.06±0.00 *b*	1.33±0.12 *b.c*
	5	23.23±0.47 *c*	1.36±0.01 *c*	1.24±0.43 *b.c*
	10	23.93±0.39 *c*	1.55±0.02 *e*	1.48±0.61 *c*
	15	19.42±0.42 *b*	1.57±0.01 *e*	1.88±0.72 *d*
	20	18.93±0.34 *a,b*	1.73±0.00 *f*	2.56± 0.80 *e*
	25	18.33±0.34 *a*	1.43±0.01 *d*	1.30±0.33 *b.c*

Values (mean ± standard deviation) with different index letters are statistically significantly different (P<0.05)

That observation may results from different ratios of mass in sprouts and seeds for soy bean than for the other germinated plants. Enzymes of seeds and grain should exhibit high tolerance to stressful conditions due to the function of seeds and grains, i.e. protection of germs against unfavourable environment conditions. The authors consciously proposed the simplification of preparation of powder for iron supplementation (patent application, 24.06.2009).

The highest specific amylolytic activity was obviously recorded for wheat-germs, especially for grains germinated in distilled water, but also for all sprouts cultured in the presented experiments. The lowest level of specific activity was observed for alfalfa sprouts over the whole experimental concentrations.

The level of enzymes activity in tested samples may also be influenced by the procedure of sprouts preparation. Using a stream of warm circulating air during the process of drying may decrease amylase activity, especially that of α-amylase. All of the samples were prepared in the same way and the process was repeatedly performed, thus changes caused by temperature processing should be comparable for the whole batch of experimented material. Also the raw material, seeds and grains before germination were dried in the same way after harvesting. It must also be noted that the concentration of extractable protein changes as a result of abiotic stress (Tab. 1–3). This conditions influence determined specific activity of studied enzymes.

The choice of a good method to determine the activity of proteases in plants is an important problem, and difficulties begin from the preparation of extracts, which need to be stable during processing and storage. An important role is also played by a variety of substances expressed in plants such as polyphenolic compounds, which may influence the determined activity. The authors decided for application of synthetic substrate (N_{α}-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride).

Changes in specific proteolytic activity are difficult to examine, because on changes in the total proteolytic activity comprise changes in neutral, alkaline and acidic protease and inhibition of endoprotease activity [Domash et al., 2008]. Only an increase in proteolytic activity for sprouts and a decrease in the activity of the trypsin inhibitor during germination are well documented [Bau et al., 1997].

Domash et al. [2008] suggested that abiotic stress induced by heavy metal ions inhibits the activity of neutral, acidic, and alkaline proteases at the early stage of germination for every examined plant (cereal crops and legumes). An expression of trypsin inhibitors is dependent on the individual tolerance to the respective stress factor and is involved in the response to stress conditions.

The changes in proteolytic activity in alfalfa sprouts are easiest to interpretation (Tab. 2). The proteolytic activity of alfalfa sprouts decreases consistently with an increase of $FeSO_4$ concentration.

The specific proteolytic activity in soy bean sprouts (Tab. 3) increases up to the concentration of $FeSO_4$ of 20 mM $FeSO_4$ and in the case of 25 mM it is lower. It may be the result of a lower tolerance of trypsin inhibitors than soy bean proteases to concentration of $FeSO_4$ in growth medium. It may also confirm the suggestion that concentration of 25 mM strongly inhibits karyokinesis of soy bean sprouts.

Changes in specific proteolytic activity of wheat-germs are presented in tab.1. Inhibition of protease activity is observed for wheat germs growing in a medium with 0–2 mM FeSO4 concentration. For higher concentrations of FeSO4 (3–5 mM) an increase of the activity in

cultured wheat germs suggested progressive inhibition of sprouts growth, progressive proteolysis and cell death. For wheat-germs the lower tolerance of trypsin inhibitors than wheat proteases to $FeSO_4$ is supposed.

Presented results are inconsistent with the results of Domach et al. [2008]. Their experiments suggested a higher resistance of Graminaceae than legumes to the effect of heavy metals present in the growth medium. Obtained results show that activity of soy bean proteolytic enzymes may be most resistant to iron in the growth medium during germination. This may be again an effect of high participation of seeds in prepared materials in comparison to the mass of sprouts, thus high participation of seeds enzymes in examined extracts.

Changes in activity of studied enzymes are difficult to examine. The authors can hypothetize different defensive mechanisms, which influence the activity. At first it should be taken into account the regulation of biosynthesis of enzyme, inhibitors and activators (cofactors) which could be activated or inhibited in species-specific concentrations of stressful factor. Next it is possible the alternative biosynthesis pathway of protein under stress conditions, in other words we can not exclude special enzyme synthesis, as it was suggested for α-amylase extracted from wheat-germs cultured in 5mM of $FeSO_4$. The preliminary experimental data indicate basically different molecular mechanisms of hydrolysis and hydrolytic enzymes activation between the tested plants.

Conclusions

The use of sprouts prepared using the presented method in food industry may influence technological properties of processed materials. Even high concentration of $FeSO_4$ in the culture medium may not inhibit the activity of studied enzymes. The specific activity of tested enzymes even may increase under stress conditions, as it was observed for amylases and proteases extracted from soy bean germinated in up to 20 mM of $FeSO_4$ solution and enzymes from wheat-germs: amylases from germs cultured in up to 2 mM or proteases from germs cultured in up to 3 mM of $FeSO_4$.

Amylases and proteases extracted from processed in presented method soy bean sprouts seem to be more stable under stress conditions induced by the presence of Fe^{2+} in the culture medium than these enzymes extracted from prepared alfalfa sprouts and wheat-germs.

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9

PROTEIN AND FAT CHANGES AND MICROBIOLOGICAL STATUS OF HAMBURGERS IN RELATION TO PACKAGING AND STORAGE CONDITIONS, AS WELL AS HEATING PROCEDURE

Introduction

Hamburger's technology is based on utilization of comminuted raw materials rich in fats. Due to this fact the quality of the hamburgers formula constituents, such as microbiological and lipids statuses should be optimised. Both groups of mentioned quality parameters can be effected by for example application of appropriate heating methods [Ou and Mittal, 2006; Rodriguez-Estrada et al., 1997]. To ensure the highest quality of hamburgers specific technologies are applied in order to reduce microbial contamination of raw materials as well as limitation of degenerative changes during production, storage and preparation for consumption [Lee et al., 2005; Gill et al., 2001; Ou and Mittal, 2006; Erdogdu et al., 2005; Mater, 2002; Sayago-Ayerdi et al., 2008; Park et al., 2004; Ozkan et al, 2004; Przybył et al., 1998; Rodriguez-Estrada et al.,1997; Feiner, 2006]. Despite leading a number of research within the above issues, due to technical and technological progress, it is still actual.

The aim of the study was the evaluation of proteins and lipids degenerative changes as well as microbiological status of beef hamburgers which were packed under vacuum or MAP stored at the temp. +3 or -3°C up to 28 days. Proteolytical changes in proteins and oxidative and hydrolitical changes in lipids were analysed in hamburgers after three different thermal treatment i.e. microwaving, steaming and baking.

Materials and methods

Materials used in the experiment were hamburgers formulated in industrial conditions with beef meat according to the commercial formula and technology. Production conditions were previously described by Szmańko et al. [2009].

Variation factors used in the experiment were as follows:

- Packaging method:
  - vacuum packaging using laminated bags PA20/PE80 (90% of air evacuation was applied),
  - packaging under inert gasses (MAP: 80% N_2, 20% CO_2) using laminated bags with two layers: MULTISEVEN 80 HS TOP (upper layer) and HIGH GLOSS FP (bottom layer).

- storage conditions:
  - chilling storage (+3°C±1°C),
  - storage at the temperature close to cryoscopic point (-3°C±°C), cryoscopic temperature for hamburgers was -2,33°C.
- Hamburgers were stored for 0, 7, 14, 21, 28 days.
- After storage hamburgers were thermally treated in order to prepare for consumption using:
  - heating in microwave oven SHARP, GRILL MICROWAVE OVEN type R-6R71, λ 2450 MHz, 1,2 kW (1). Hamburgers were thermally treated for 2 min 10 sec.
  - heating in steam at the temp. 100°C (2) for 6 min.,
  - baking in an electric oven at the temp. 170°C (3) for 7 min.

All samples were heated up to temp. 70°C was reached in the geometrical centre of the product. The temperature after removing from the oven increased to 73°C ±1°C. Processing conditions were established in preliminary experiment.

Collected samples were then subjected to analysis of soluble protein content by homogernisation of 5 g of the product with 20ml of 1 M NaCl solution, centrifugation at 1 900 x g for 15 min and protein content evaluation in supernatant according to Kjeldahl method [PN-75/ A-04018]. Free amine groups content was evaluated with method described by Kuchro et al. [1983] with the modifications of Chrzanowska et al. [1993]. In order to lipids status analyses, such as peroxide value, acidic value and TBA, experimental hamburgers were heated in dryer at 50°C for 12 h. Following lipids extraction was performed by the addition of petroleum ether and shaking for 12 h at room temperature. Obtained lipids, after ether removal, was subjected to further analyses [PN ISO 1444:2000], peroxide value [PN-ISO 3960:1996] acidic value [PN-ISO 660:1998/Az1:2000], level of thiobarbituric reactive substances (TBA) according to Pikul et al. [1989]. Water activity (a_w) was analysed using NOVASINA HUMIDAT–IC equipment.

Microbiological status analyses included: general number of aerobic bacteria [PN-A-82055-6:1994]; molds and yeasts [PN-A-82055-16:1994]; number of lactic acid bacteria [PN-A-82055-17:1997]; *Enterobacteriacea* [PN-64/A-82054]; *Salmonella* [PN-A-82055-8:1994]; *Escherichia coli* [PN-ISO 6391:2000]; coagulase-positive staphylococcus [PN-A-82055-9:1994]; proteolytic bacteria analysed on Smith-Goodnc base incubated at temp. 37°C for 24 h. The results collected in the study were statistically analysed using STATGRAPHICS ver. 5.0 at the probability level $p>0.05$

Results and disscussion

Results collected in the study revealed that storage period influenced the amount of soluble proteins in beef hamburgers. Significant decrease in soluble protein content was analysed after 14 days of chilled storage in relation to the material stored for 7 days (Tab. 1). Packaging methods as well as storage temperature did not effect the level of soluble protein in the hamburgers (Tab. 2), whereas there was influence of applied heating methods (Tab. 3). As was predicted, the most negative effect on proteins solubility had heating by microwaves, probably due to the shortest time of the process. No differences were observed in protein solubility between steamed and baked hamburgers. Similar results were reported by Rodriguez-Estrada et al. [1997], whose showed only slight effects of different thermal

treatment methods on protein degradation in hamburgers. Decreasing in protein solubility during storage of the hamburgers was connected with lower water holding capacity [Szmańko et al., 2009].

Fresh (not stored) experimental hamburgers were characterised by an average content of free amine groups on the level of 2 800 µg Gly/1g protein (Tab. 1). During storage free amine groups content increased significantly up to almost 2 900 µg Gly/1g protein after 14 days and up to 4 500 µg Gly/1g protein after 21 days. No significant influence on free amine groups content was observed in relation to packaging methods and storage temperature (Tab. 2). Tendency to lower analysed chemical groups content occurred when store vacuum packed hamburgers at the temperature close to cryoscopic point (-3°C). Proteins present in beef hamburgers were not effected by heating method (Tab. 3). However, the characteristic trend of correlation between heating time and amount of free amine groups was observed. Microwaved hamburgers were characterised by lower content of free amine groups in relation to steamed and baked products.

Table 1

The effect of storage period on lipids and protein changes in thermally treated hamburgers, n = 36

Indicator		Storage period [days]				
		0	7	14	21	28
Protein solubility [%]	X	0.32^{c*}	0.33^{c}	0.31^{b}	0.28^{a}	0.30^{b}
	sd	0.06	0.06	0.04	0.05	0.04
Free amine groups content [µg Gly/1g protein]	X	2801^{b}	2493^{a}	2890^{b}	4435^{c}	4404^{c}
	sd	93	105	98	87	112
Peroxide value [ml 0.002 N $Na_2S_2O_3$/1 g lipids]	X	1.0^{a}	1.0^{a}	1.22^{b}	1.86^{c}	3.17^{d}
	sd	0.08	0.10	0.11	0.17	0.20
Acidic value [ml 0.1 N KOH/1 g lipids]	X	2.0^{a}	2.08^{a}	2.61^{b}	3.0^{c}	3.08^{b}
	sd	0.04	0.08	0.01	0.05	0.02
TBA value	X	0.891^{a}	0.861^{a}	1.00^{a}	1.000^{a}	1.440^{b}
	sd	0.05	0.01	0.02	0.01	0.02

*a, b,c – Means within a row do not differ significantly (P>0.05) if they have a common letter or if they have no letters.

Table 2

The effect of storage temperature and packaging methods on lipids and protein changes in thermally treated hamburgers, n = 90

Indicator		Packaging method		Storage temperature [°C]	
		vacuum	MAP	+3	-3
Protein solubility [%]	X	0.31	0.30	0.31	0.30
	sd	0.04	0.2	0.04	0.1
Free amine groups content [µg Gly/1g protein]	X	3378	3431	3470	3339
	sd	59	62	71	68
Peroxide value [ml 0.002 N $Na_2S_2O_3$/1 g lipids]	X	1.73^{b}	1.57^{a}	1.79^{b}	1.51^{a}
	sd	0.04	0.18	0.21	0.03
Acidic value [ml 0.1 N KOH/1 g lipids]	X	2.52	2.59	2.56	2.56
	sd	0.03	0.01	0.03	0.01
TBA value	X	1.056	1.022	1.00	1.08
	sd	0.04	0.01	0.03	0.03

Table 3

The effect of heating methods on lipids and protein changes in thermally treated hamburgers, n = 60

Indicator		Heating method		
		microwaving	steaming	baking
Protein solubility [%]	X	0.32^{b}	0.30^{a}	0.30^{a}
	sd	0.05	0.38	0.27
Free amine groups content [μg Gly/1g protein]	X	3349	3364	3501
	sd	73	68	57
Peroxide value [ml 0.002 N $Na_2S_2O_3$/1 g lipids]	X	1.58^{a}	1.57^{a}	1.80^{b}
	sd	0.05	0.27	0.30
Acidic value [ml 0.1 N KOH/1 g lipids]	X	2.47^{a}	2.62^{b}	2.58^{b}
	sd	0.03	0.01	0.01
TBA value	X	0.983^{a}	1.017^{b}	1.117^{b}
	sd	0.04	0.06	0.03

An important factor in the quality of fat containing products is extension of oxidation and hydrolytic changes. Those processes can be induced by many factors during processing and storage; among other by thermal treatment [Park et al., 2004; Rodriguez-Estrada et al., 1997]. Storage of beef hamburgers for more than 7 days resulted in continuous increasing of peroxide value (Tab. 1), significant increase was observed after 14 days of storage. However, up to 21 days analysed changes did not limit the consumption usefulness. Hamburgers stored for 28 days reached the shelf boundary. During the whole storage period higher dynamic of peroxide value changes was observed in vacuum packed products as well as stored at +3°C (Tab. 2). Heating methods influenced lipid oxidation processes analysed in beef hamburgers (Tab. 3). Lower oxidation changes was observed in microwaved and steamed hamburgers comparing to baked products.

Hydrolytical changes of lipids was analysed in the hamburgers stored for more than 7 days (Tab. 1). The dynamic of analysed changes was stable during the whole storage period. Higher dynamic of hydrolytical changes was observed after 14 days of storage. No differences in hydrolytical changes were analysed in relation to packaging method as well as storage temperature. Heating methods applied in the study significantly influenced hydrolytical processes of lipids present in beef hamburgers (Tab. 3). Microwaving caused lower hydrolytical changes in the hamburgers in comparison to steaming and baking.

The dynamic of TBA changes in beef hamburgers was to that found in the case of peroxide and acidic values. Significant increase in the level of malondialdehyde was observed in the material stored for more than 28 days (Tab. 1). Packaging methods as well as storage conditions in means of temperature did not effect TBA value (Tab. 2). As was predicted heating methods significantly influenced oxidative changes in beef hamburgers. The lowest TBA value was analysed in microwaved hamburgers (Tab. 3), whereas steamed and baked products were characterized by higher extension of lipid oxidation processes. The aspect of degenerative changes in hamburgers lipids is well known, and therefore attempts are being made to counteract these undesirable processes including application of natural antioxidants or innovating packaging technology [Komatsu, 2001; Park et al., 2004; Mater, 2002; Sayago-Ayerdi et al., 2009].

Next parameter potentially affecting durability of meat products is water activity. In presented study only packaging methods had an influence on a_w analysed in beef hamburgers. Slightly lower water activity was measured in the hamburgers stored under modified atmosphere (MAP) (0.9408) in relation to vacuum packed products (0.9414).

Microbiological status of beef hamburgers was analysed in fresh and stored products without additional thermal treatment. As was predicted microbiological contamination of the hamburgers increased during storage.

General number of aerobic bacteria analysed in beef hamburgers stored at 3°C for 14 days was on the level of 10^6 cfu/g (Tab. 4). The amount of this type of bacteria was not affected by packaging methods applied for the hamburgers. Storage conditions i.e. temperature, significantly influenced the number of aerobic bacteria in beef hamburgers. Products stored for 28 days at the temperature close to cryoscopic point (-3°C) were characterised by the number of analysed microorganisms on the level of 10^5 cfu/g.

Table 4

General number of aerobic bacteria [cfu/g], n = 5

Storage temperature [°C]	Packaging method	Storage period [days]				
		0	7	14	21	28
+3	vacuum	4.2×10^4	2.2×10^4	2.8×10^6	3.1×10^6	2.1×10^7
-3	vacuum	4.2×10^4	1.6×10^4	4.6×10^5	2.0×10^5	4.6×10^5
+3	MAP	4.3×10^4	2.0×10^4	4.0×10^6	7.0×10^6	1.4×10^7
-3	MAP	4.3×10^4	1.1×10^4	3.7×10^5	1.0×10^5	4.9×10^5

Up to 21 days of storage the amount of molds and yeasts in beef hamburgers was on the level of 10^2 cfu/g (Tab. 5). The hamburgers stored at -3°C were characterised by slightly lower level of those microorganisms. Dynamic increase in molds and yeasts number was observed in the hamburgers stored for 28 days at the temperature of +3°C (of about 2 times), whereas less increase was observed in products kept at -3°C (of about 1 time). Packaging methods did not influence the number of analysed microorganisms.

Table 5

Moulds and yeast number [cfu/g], n = 5

Storage temperature [°C]	Packaging method	Storage period [days]				
		0	7	14	21	28
+3	vacuum	8.3×10^3	3.1×10^2	2.4×10^2	3.7×10^2	2.1×10^4
-3	vacuum	8.3×10^3	2.4×10^2	3.1×10^2	3.1×10^2	2.0×10^3
+3	MAP	8.8×10^3	5.3×10^2	1.7×10^2	3.4×10^2	2.7×10^4
-3	MAP	8.8×10^3	4.7×10^2	2.4×10^2	1.4×10^2	1.0×10^3

Control hamburgers were characterised by the similar number of *Enterobacteriaceae* in range of $2\text{-}4 \times 10^2$ cfu/g (Tab. 6). Storage of the hamburgers for 28 days at 3°C resulted in significant decrease in number of analysed bacteria. Beef burgers stored at the temperature close to cryoscopic point were *Enterobacteriaceae* free.

Table 6

Enterobacteriaceae [cfu/g]

Storage temperature [°C]	Packaging method	Storage period [days]				
		0	7	14	21	28
+3	vacuum	2.1×10^2	2.5×10^2	2.4×10^2	2.1×10^2	8.0 x 10
-3	vacuum	2.1×10^2	–	–	–	–
+3	MAP	3.7×10^2	2.7×10^2	3.7×10^2	9.0×10^2	–
-3	MAP	3.7×10^2	–	–	–	–

Lactic acid bacteria was not detected in the hamburgers stored for 7 days at both analysed temperatures packed under MAP or vacuum (Tab. 7). From day 14 of storage of the hamburgers at +3°C similar number of lactic acid bacteria was detected. Analysed microorganisms were not present in products stored at the temperature of -3°C.

In all analysed beef hamburgers *Salmonella*, *Escherichia coli*, coagulase-positive staphylococcus as well as proteolytic bacteria were not detected within the whole storage period.

Table 7

Lactic acid bacteria [cfu/g]

Storage temperature [°C]	Packaging method	Storage period [days]				
		0	7	14	21	28
+3	vacuum	–	–	2.2×10^2	3.7×10^2	2.9×10^2
-3	vacuum	–	–	–	–	–
+3	MAP	–	–	3.1×10^2	2.4×10^2	4.2×10^2
-3	MAP	–	–	–	–	–

Ciftcioglu et. al. [2008] reported that *Escherichia coli* 0157:H7 was characterised by higher survival in comminuted meat and hamburgers at 4°C and -18°C than *in vitro*, thus presence of this bacteria should be monitored in refrigeration chain of the hamburgers. Ou and Mittal [2006] developer a mathematical model enabling the effective inactivation of *Escherichia coli* 0157:H7, *Listeria innocua* and *Salmonella serotypes* in the hamburgers by application thermal treatment at the temperature of 160°C for 115-293 sec. Moreover, Gill et al. [2001] proposed short pasteurisation at 85°C of meat used for hamburgers preparation to ensuring higher microbiological status of final products. However, Erdogdu et al. [2005] pointed out the need to strike the compromise in determining the conditions of heat treatment between the expected microbiological safety and rheological properties and weight losses.

The consequences of degenerative changes in proteins of beef hamburgers were Lower protein solubility and higher free amine groups content in products stored for 21 and 14 days, respectively. Microwaved hamburgers were characterized by higher protein solubility comparing to steamed and baked products. Packaging methods and storage temperature did not influence soluble protein content. Vacuum packed hamburgers as well as stored at the temperature close to cryoscopic (-3°C) were characterised by lower free amine groups content. Increases in acidic value, peroxide value and TBA were noticed respectively from 14, 21 and 28 days of storage of the hamburgers. Microwaved products were characterized by lower dynamic of oxidative changes than steamed and baked ones. Lower dynamic of oxi-

dative processes was also analysed in beef hamburgers packed under MAP and stored at –3°C, in comparison to those vacuum packed and stored at +3°C. The highest level of malondialdehyde was observed in baked hamburgers. General number of aerobic bacteria in beef burgers stored up to 28 days at -3°C, despite the packaging methods, was on the level of about $5x10^5$ cfu/g, whereas in the hamburgers kept up to 14 days at +3°C h was between 2.8 and 4.0 x 10^6 cfu/g. Up to 21 days of storage the amount of molds and yeasts in the hamburgers was stable despite the packaging methods and storage temperature. Significant increase in the number of analysed microorganisms was observed after 28 days of storage at -3°C and +3°C, respectively of about 1 and 2 times. *Enterobacteriaceae* and lactic acid bacteria were not detected in beef hamburgers stored at temp. -3°C despite the packaging methods. In all experimental hamburgers no *Salmonella*, *Escherichia coli*, coagulase-positive staphylococcus and proteolytic bacteria were detected. It can be concluded that storage of the hamburgers at the temperature close to cryoscopic point (-3°C) resulted in lower dynamic of degenerative changes in lipids and proteins as well as higher microbiological safety.

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10

ESTIMATION OF CHEMICAL COMPOSITION AND COLOUR OF CORNELIAN CHERRY COMPOTES

Introduction

The fruits of cornelian cherry (*Cornus mas* L.) are stone fruits with shape from spherical to oval. Their size is diversified. The weight of single fruits ranges from 1,4 to 5.2g [Demir & Kalyoncu, 2003; Pirlak et al., 2003], or even, as reported by Yilmaz et al. [2009] 9.2 g, while the diameter – from 0.9 to 19.0 mm [Tural & Koca, 2008]. Flesh/stone ratio, depending on the size and shape of fruits ranges from 6 to 12. The color of fruits may be yellow, pink, red, cherry, or even black, as chokeberry. It is anthocyanins that are responsible for the red color of fruits. Among them, two dominating: galactosides of cyanidin and pelargonidin were identified. The rest of anthocyanins includes rutinosides of cyanidin and of pelargonidin and, in nearly untraceable quantities, galactoside of delphinidin. The rind of fruits is darker than the flesh and contains 4 or 5 times more anthocyanins [Klimenko, 2004].

Cornelian cherry fruits are succulent, with pleasant sweet-sour taste and delicate aroma. Soluble solids content is in a wide range from 11.5 to 24% [Demir & Kalyoncu, 2003; Guleryuz et al., 1998; Pirlak et al., 2003]. The fruits contain from 4 to 15% of total sugar [Guleryuz et al., 1998; Tural & Koca, 2008], among which equal quantities of glucose and fructose dominate. Titratable acidity amounts from 1.1% to 4.7% [Guleryuz et al., 1998; Pirlak et al., 2003; Tural & Koca, 2008].

Cornelian cherry fruits are valuable source of active compounds as vitamin C, polyphenols, or, mentioned earlier, anthocyanins. Because of their attractive chemical composition and intensive color, they may be processed into many products, e.g. liqueurs, juices, jam, purées and compotes. The latter seem to be very attractive because of appearance and taste of cornelian cherry fruits.

Cornelian cherry products, including compotes, depending on the variety, chemical composition and fruit color, as well as way of processing and storage conditions may exhibit large differentiation.

Compotes are underestimated products, which are recently supplanted by juices and drinks. The raw material intended for compote production should have dense flesh, uniform look, correct shape and color as well as proper aroma and good taste.

The taste of compotes and other organoleptic features are defined by extract to acidity ratio. As given by Berdowski [1991] for compote extract equal to 18%, the acidity should be 0,3%. It is assumed that the increase of acidity about 0,1% should be accompanied by increase of extract about 1%. In the case of using stone fruits for compote production, stones should be removed. Unfortunately, this changes unfavorably the appearance of

fruits, therefore producers often abandon removing stones, although the presence of stones in compotes, in particular cherry ones, reduces their organoleptic and health features.

The aim of this work was to estimate the chemical composition, antioxidant activity, and color of compotes from cornelian cherry, after 1, 6, and 9 months of storage in the room temperature.

Materials and methods

To get compotes, we use fruits of cornelian cherry from Arboretum and Institute of Physiography in Bolestraszyce. The fruits were harvested fully ripe, from five distinct bushes. Stewed fruits were prepared in two variants (W1 – fruits with stones, and W2 – fruits without stones) according to the standard for stewed cherries.

Preparation of cornelian cherry compotes

Cornelian cherry fruits from five distinct bushes were selected and washed. Next, each sample was divided into two parts – from fruits constituting one part stones were removed by hand, while the second one was left with stones. Stones constituted 30% of fruit weights. Fruits with stones (100 g) and without stones (70 g) were put into jars (205 mL and 130 mL, respectively), and poured with hot (70ºC) water with sugar. The fruits were entirely covered – the ratio of fruits to syrup was 1:1. The amount of sugar was calculated in such a way that soluble solids content of compotes was 24%. After closing, the jars were pasteurized (85ºC, 30 minutes) and then cooled down to about 20ºC. Obtained products were stored in the dark place in room temperature (around 25ºC) and analyzed after obtaining equal concentrations of soluble solids content in fruits and syrup, after 1 month (control sample) and after 6 and 9 months.

HPLC analysis of anthocyanins

Anthocyanins were determined using Dionex (USA) HPLC system equipped with diode array detector model Ultimate 3000, a quaternary pump LPG-3400A, autosampler EWPS-3000SI, thermostated column compartment TCC-3000SD and controlled by Chromeleon v.6.8 software. A reversed phase Atlantic T3 (250 mm x 4.6 i.d., 5 μm) column (Waters, Ireland), guard column and Atlantis T3 (20 x 4.6 i.d, 5 μm) guard column (Waters, Ireland) were used. Solvents that constituted the mobile phase were: A (4.5% formic acid) and B (acetonitrile). The applied elution conditions were: 0–1 min 5% B isocratic; 1–6, min linear gradient from 5% to 10% B; 6–26 min, linear gradient 10–20% B; 26–33 min, linear gradient from 20% to 100% B; and finally, washing and reconditioning of the column was done. The flow rate was 1 mL/min, and the injection volume was 20 μL. The column was operated at 30°C. Anthocyanins were monitored at 520 nm.

Antioxidant activity analysis

Ferric reducing antioxidant power (FRAP) was measured according to Benzie and Strain [1996]. DPPH (1,1-didhenyl-2-picrylhydrazyl radical) assay was carried out as described by Yen and Chen [1995]. $ABTS^{\bullet+}$ (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay was done according to the method of Re et al. [1999]. Results of antioxidant activity are expressed in μmol Trolox equivalents (TE)/1 g.

Colour analysis

The colour of compote (fruit and syrup) was measured using Colour Quest XE colorimeter (Hunter Associates Laboratory, Inc., USA). The instrument was calibrated with the white standard tile (L^*=93.92; a^*=-1.03, b^*=0.52). Samples of syrup (fruit) were placed in the 1 cm (2 cm) path length glass cell (Hellma, Germany) and L^*, a^*, b^*, *haze* were measured in triplicate in the total transmission (reflection) mode using Illuminant D_{65} and 10° observer angle.

Analyses

Dry matter (DM) was determined according to polish standard (PN-90/A-75101/03). Soluble solids content (SSC) was determined with refractometer (PN-90/A-75101/02). Titratable acidity (TA) (as malic acid) was determined according to PN-90/A-75101/04. Vitamin C was determined using the Tillmans method (PN-90/A-75101/1) prior to adsorption of pigments into a C18 Sep-Pack cartridge (Waters Associates, Milford, MA, USA). The total polyphenol (TPol) content was measured with the Folin-Ciocalteu reagent at 765 nm with gallic acid (GEA) as a standard, according to Gao [2000]. The results were calculated as mg of GEA in 100 g.

All determinations were performed in triplicate. Statistical analyses were performed using Statistica 8.0 (StatSoft, Poland). Significant differences ($P < 0.05$) between mean values for five compotes from different cornelian cherry bushes were evaluated by using one-way ANOVA with Duncan test. Correlation coefficients (r) were calculated.

Results and discussion

The acidity and contents of dry matter, extract, and vitamin C in compotes obtained from fruits with (W1) and without (W2) stones, after one month of storage in room temperature is shown in Table 1. After one month from compotes preparation, the concentrations of both acid and sugars fruits and syrup equalized.

There were no statistically significant differences in soluble solids content and acidity, between fruits and syrup. However, there were significant differences between compote variants. In compotes with and without stones the SSC was 24 and 26%, respectively, and TA – 1.1 and 1.3%. Significantly higher level of SSC and TA in compotes without stones (W2) is caused by different values of flesh to syrup ratio, with constant fruits to syrup ratio 1:1. In compotes (W1), because the presence of stones, there had to be more syrup, in order to cover all fruits. According to suggestions of Berdowski [1991] for obtained extract the total acidity should be lower by 0.2%. The SSC to TA ratio is not that suggested by Berdowski [1991] for fruit compotes, because the acidity of cornelian cherry fruits is relatively high, comparing to other fruits [Guleryuz et al., 1998; Pirlak et al., 2003; Tural & Koca, 2008]. Therefore, if following Berdowski, one should add more sugar to syrup, which could unfavorably affect the taste of the product. Depending on variety, acidity of cornelian cherry fruits is between 1.1 and 4.7% [6, 13, 23,], and SSC – between 11.5 and 24% [Demir & Kalyoncu, 2003, Guleryuz et al., 1998, Pirlak et al., 2003]. Total acidity in domestic fruits ranges form 0,6 (pears) to 3,0 g/100 g (black currants), whilst in drupes – from 0,8 to 1,3 g/100g [Zadernowski & Oszmiański, 1994.]. Mean dry matter for fruits was from 27.4% in compote with stone, to 28.4% in compote without stone, and it was significantly higher

than in syrup (24.5–26.1%). High level of dry matter is caused by both its high level in fruits, and by the amount of sugar added to syrup. Cornelian cherry fruits have high content of dry matter [Tural & Koca, 2008] – higher than fruits of sour cherry, strawberry or raspberry, [Zadernowski & Oszmiański, 1994]. The content of vitamin C in compotes ranged from w 2 mg/100 g (min) to 14 mg/100 g (max) and that level depended on fruits used (fruits from different bushes). Cornelian cherry fruits, depending on variety, contain large amounts of vitamin C, from 16 to 112 mg/100g [Demir & Kalyoncu, 2003, Guleryuz et al., 1998, Pantelidis et al., 2007, Pirlak et al., 2003, Tural & Koca, 2008, Yilmaz et al., 2009.]. However, as a result of thermal processing, i.e. pasteurization of compotes, the concentration of vitamin C lowered. Mean content of vitamin C in cornelian cherry compotes was about 5 times lower than in cornelian cherry juices obtained by Gasik et al. [2008]. In fruits there was slightly more vitamin C than in syrup, but this difference was not statistically significant. Similarly, we did not observe significant differences between compote variants. From this we conclude that possible losses of vitamin C during removing of stones (W2) gave comparable effect to diluting this vitamin in compote (W1) by slightly greater addition of syrup.

Table 1

Dry matter (DM), soluble solids content (SSC), titratable acidity (TA) and vitamin C (Vit C) of cornelian cherry compote after 1 month of storage at room temperature

Variant of compote	Part of compote		DM [%]	SSC [%]	TA [%]	Vit C [mg/100mg]
Fruit with stone (W1)	Fruit	Mean ± SD	27.37±2.13ab	24.49±1.72b	1.14±0.21b	7.64±3.91a
		Max	29.36	26.30	1.43	14.13
		Min	24.83	22.50	0.92	3.84
	Syrup	Mean ± SD	24.52±2.32c	24.26±2.24b	1.08±0.15b	6.28±4.47a
		Max	26.84	26.90	1.24	13.62
		Min	21.525	21.5	0.88	2.04
Fruit without stone (W2)	Fruit	Mean ± SD	28.45±0.88a	26.08±0.95a	1.32±0.20a	9.18±4.07a
		Max	29.10	27.05	1.55	14.30
		Min	26.91	24.50	1.07	3.02
	Syrup	Mean ± SD	26.135±0.96b	26.00±0.85a	1.319±0.19a	7.49±4.43a
		Max	26.96	26.60	1.55	14.14
		Min	24.48	24.50	1.08	2.99

Mean values within a columns with different letters are significantly different at $P < 0.05$.

Quantitative and qualitative results of anthocyanin compounds, assayed using HPLC method, are shown in Table 2. Total contents of anthocyanins, after 1 month of storage was on average from 48.3 mg/100 g in compotes from fruits with stone to 58.8 mg/100 g in compotes from fruits without stone. Main anthocyanins were galactosides pelargonidin and cyanidin [Seeram et al., 2002], which constituted 95% of red colorants in compotes from fruits without stone and 84% – in compotes from fruits with stone. It was pelargonidin that dominated in tested compotes, with the amount 2 to 4 times larger than cyanidin. The maximum amount of pelargonididin-3-*O*-galactoside and cyaniding-3-*O*-galactoside was in

fruits from compote without stone (W2) and equaled, respectively, to 39.60 mg/100 g and 29.56 mg/100 g, while minimum – in fruits from compote with stone (W1) and was, respectively, 17.79 mg/100 g and 7.82 mg/100 g. While analyzing mean results, we have observed that among rutinosides there was slightly less of pelargonidin (5%) than cyanidin (6-7%), in relation to the total amount of anthocyanins. Delphinidin-3-*O*-galactoside constituted less than 1% of total anthocyanins.

Table 2

Anthocyanins, total anthocyanins (TAnt), total polyphenols (TPol), antioxidant activity (DPPH, ABTS, FRAP) of cornelian cherry fruit from compote after 1 month of storage at room temperature

	Fruit			
	Fruit with stone (W1)		Fruit without stone (W2)	
	Means ± SD	Range	Means ± SD	Range
Df -3-gal (mg/100g)	0.33±0.23a	0.00-0.64	0.56±0.20a	0.43-0.92
Cy -3-gal (mg/100g)	16.30±7.13a	7.82-27.40	21.14±9.19a	9.90-29.56
Cy-3-rut (mg/100g)	2.82±1.76a	0.76-5.10	4.41±1.80a	1.97-6.85
Pg-3-gal (mg/100g)	26.62±5.92a	17.76-33.80	29.60±6.11a	24.55-39.60
Pg-3-rut (mg/100g)	2.28±1.46a	0.46-3.93	3.11±1.25a	2.08-4.85
TAnt (mg/100g)	48.34±9.67a	39.73-64.22	58.83±6.50a	50.69-68.73
TPol (mg/100g)	379.1±52.5a	447.9 - 321.2	285.5±33.0b	338.5 - 206.1
DPPH (μM trolox/1g)	17.98±0.75a	19.01 - 17.31	15.10±1.27b	16.42 - 13.17
ABTS (μM trolox/1g)	31.30±3.96a	36.41 - 26.39	27.08±2.69a	30.44 - 22.96
FRAP (μM trolox/1g)	17.56±2.37a	21.41 - 15.67	14.13±1.88b	15.80 - 10.96

Mean values within a verses with different letters are significantly different at $P < 0.05$.
Df-3-gal – Delfinidin-3-*O*-galactoside, Cy-3-gal – Cyjanidin-3-*O*-galactoside, Cy-3-ryt – Cyjanidin-3-*O*-rutinoside, Pg-3-gal – Pelargonidin-3-*O*-galactoside, Pg-3-rut – Pelargonidin-3-*O*-rutinoside. Total anthocyanins=total amount (sum) of anthocyanins determined by HPLC.

In Table 2 it is also shown the concentration of polyphenols and antioxidant activity assayed by DPPH, ABTS and FRAP methods, of fruits from compotes after 1 month of storage. Total polyphenols content was in large interval from 206 to 448 mg/100 g. It has been observed that the amount of polyphenols, contrary to anthocyanins and vitamin C, was a significantly higher in compotes made from fruits with stone than in compotes made from fruits without stone. Similar results were obtained by the authors in earlier investigations [Kucharska et al., 2007]. It is probably caused by migration of active compounds from fruit stones to flesh. Those compounds react with Folin-Ciocalteu reagent.

Fruits of cornelian cherry are rich source of polyphenols. Tural & Koca [2008] gives the range of their concentrations in fresh fruits from 281 to 579 mg/100 g. However, as a result of processing of cornelian cherry fruits, the amount of polyphenols may be changed. The level of those changes may be influenced by many factors, like temperature and heating time, activity of enzymes, access of oxygen, light, and many others. In cornelian cherry juices obtained from pulp depectinized for various time (duration) Gasik et al. [2008] assayed total plyphenols on the level 251–261 mg/100 g.

Table 2 contains results of antioxidant activity given in μM Trolox/1 g. Higher activity ($P < 0.05$) was noted for fruits from compotes prepared according W1 variant than according to W2 variant. Probably, it is the results of transfer of active compounds from stones to

flesh during storage of compotes. Mean values of radical DPPH scavenging and of ferric reduction (FRAP) were comparable and were respectively 17.98 μM Trolox/1 g for W1 and 15.10 μM Trolox/1 g for W2, and 17.56 μM Trolox/1 g for W1 and 14.13 μM Trolox/1g for W2. Two times larger values of activity were obtained for radical cation $ABTS^{\bullet+}$ (31.3 μM Trolox/1 g – 27.08 μM Trolox/1g) in comparison to values obtained using DPPH and FRAP. Probably it results from different (selective) activity of active compounds against mentioned radicals. Gasik et al. [2008] investigating cornelian cherry juices obtained slightly higher values of the activity, using DPPH (18 – 20.5μM Trolox/1g).

Table 3 shows concentrations of anthocyanins, polyphenols, and vitamin C, as well as values of antioxidant activity of fruits and syrup of compotes after 6 and 9 months of storage. During compote storage, the degradation of active compounds occurred, and what follows the antioxidant activity was lowered. Anthocyanin colorants were subject to degradation to the highest degree. Their losses after 6 months were 71–78% and after 9 months – 83–87%. Higher losses of colorants have been observed in compotes prepared according to variant W1 than W2. Also, particular anthocyanins were characterized by different stability. Both the aglycon and associated sugar influenced the stability of anthocyanins during storage of compotes. Pelargonidins were more stable than cyanidins and rutynosides than galactosides. Lower losses during storage were observed in contents of vitamin C and plyphenols. After 6 months the amount of vitamin C decreased by 53% in compote from fruits with stone, while in compote from fruits without stone – by 64%. In turn, losses of total polyphenols after 6 months of storage were 23–32%. During next 3 months of storage, vitamin C underwent almost total degradation and only trace amounts were assayed. At the same time the amount of total polyphenols (assayed with Folin-Ciocalteu reagent), both in fruits and in syrup increased. Initial decrease and following increase of assayed polyphenols during compote storage may be attributed to their initial degradation and then developing of polymers, exhibiting activity against Folin-Ciocalteu reagent. Similarly to assayed polyphenols behaved the antioxidant activity of compotes. Antioxidant potential during first six months of storage decreased, and during next few months slightly increased, which may be explained by higher activity of developing polymers. Similar observations were done by other authors [Klimczak et al., 2007; Piljac-Zegarac et al., 2009].

Many authors [Deighton et al., 2000; Wang & Ciao, 2000; Pantelidis et al., 2007], found linear correlation between antioxidant activity and total polyphenols in fruits. However, the same authors reported that the anthocyanins had a minor influence on antioxidant capacity. In the present study, DPPH, FRAP and ABTS activity results were in good correlation with total polyphenols ($r = 0.77$, 0.72, and 0.67, respectively). Between the total anthocyanin contents and ABTS method results correlation was found ($r = 0.62$), but we did not observe correlation with results obtained using DPPH, FRAP methods. We did not find positive correlation between antioxidant activity and vitamin C in cornelian cherry compote (Table 4), which is in agreement with results obtained by other researchers [Kalt et al., 1999; Deighton et al., 2000; Pantelidis et al., 2007].

Table 3

Anthocyanins, total anthocyanins (TAnt), total polyphenols (TPol), antioxidant activity (DPPH, ABTS, FRAP) of cornelian cherry compote after 6 and 9 months of storage in room temperature

	Fruit with stone compote (W1)				Fruit without stone compote (W2)			
	Fruit		Syrup		Fruit		Syrup	
	Means ± SD	Range	Means ± SD	Range	Means ± SD	Range	Means ± SD	Range
				6 months				
Df -3-gal	T	–	0.00±0.00a	0.00–0.00	T	–	0.00±0.00a	0.00–0.00
Cy -3-gal	3.04±1.55a	1.56–5.35	0.64±0.41b	0.25–1.26	4.65±2.48a	2.22–8.72	0.94±0.49b	0.58–1.78
Cy-3-rut	0.64±0.49ab	0.00–1.15	0.17±0.13b	0.00–0.32	1.04±0.72a	0.21–1.97	0.25±0.18b	0.02–0.47
Pg-3-gal	6.33±2.37b	3.69–8.76	0.92±0.25c	0.53–1.14	9.04±2.72a	5.63–12.12	1.49±0.57c	0.89–2.35
Pg-3-rut	0.66±0.49ab	0.00–1.22	0.12±0.08b	0.00–0.20	0.99±0.68a	0.14–1.72	0.20±0.15b	0.01–0.37
TAnt	10.68±3.74b	5.38–14.59	1.84±0.77c	0.83–2.86	15.75±4.35a	10.83–20.63	2.89±0.94c	1.71–3.86
TPol	289.5±42.1a	354.0–247.2	194.0±11.0bc	208.5–181.4	231.2±28.0b	276.3–204.3	163.0±21.8c	190.5–142.2
Vit C	3.54±2.14a	6.73–1.29	3.23±2.27a	7.16–1.3	6.26±3.19a	8.55–3.02	5.50±3.55a	7.16–1.30
DPPH	15.48±1.88a	17.52–13.47	10.92±1.02b	12.04–9.73	12.84±1.91b	15.50–10.54	8.76±1.29c	10.33–7.06
ABTS	26.71±3.94a	31.83–22.64	18.60±2.66b	21.15–14.68	23.34±2.81a	26.57–19.89	15.20±3.04b	18.07–11.30
FRAP	15.90±2.55a	18.95–12.79	12.54±2.35b	15.43–10.13	13.27±2.16ab	16.10 – 10.49	10.30±2.23b	12.82–7.65
				9 months				
Df -3-gal	0.00±0.00	0.00–0.00	0.00±0.00	0.00–0.00	0.00±0.00	0.00–0.00	0.00±0.00	0.00–0.00
Cy -3-gal	1.55±1.47ab	0.00–3.93	0.39±0.36b	0.00–0.95	2.41±1.69a	0.00–4.58	0.69±0.46b	0.00–1.26
Cy-3-rut	0.56±0.32ab	0.21–0.95	0.16±0.08c	0.04–0.25	0.86±0.42a	0.41–1.46	0.29±0.11bc	0.15–0.44
Pg-3-gal	3.18±2.39ab	0.00–5.56	0.57±0.35b	0.00–0.84	5.40±4.31a	0.00–10.78	1.15±0.81b	0.00–2.23
Pg-3-rut	0.87±0.81ab	0.14–2.23	0.17±0.13b	0.06–0.40	1.43±1.28a	0.31–3.55	0.35±0.30ab	0.12–0.85
TAnt	6.15±2.94b	2.86–9.34	1.29±0.55c	0.63–2.15	10.10±4.45a	5.00–14.57	2.48±0.77c	1.29–3.29
TPol	322.4±37.9a	359.3–275.0	264.3±34.32b	312.7–223.1	248.6±28.9b	287.5–218.6	235.3±41.8b	304.7–197.9
Vit C	T	T	T	T	T	T	T	T
DPPH	21.92±2.90a	24.36–17.11	12.95±2.39bc	16.36–10.74	16.31±2.73b	19.17–13.56	12.20±2.73c	16.62–9.79
ABTS	27.52±3.51a	31.33–23.03	12.51±2.31b	16.24–10.40	22.94±4.84a	30.10–17.96	12.09±2.58b	14.87–8.48
FRAP	17.50±2.09a	19.82–14.15	17.45±3.35a	22.13–14.19	13.91±2.69a	16.74–10.01	15.67±3.36a	21.10–12.99

Mean values within a verses with different letters are significantly different at $P < 0.05$. T – trace amount.

Df-3-gal – Delfinidin-3-*O*-galactoside, Cy-3-gal – Cyjanidin-3-*O*-galactoside, Cy-3-ryt – Cyjanidin-3-*O*-rutinoside, Pg-3-gal – Pelargonidin-3-*O*-galactoside, Pg-3-rut – Pelargonidin-3-*O*-rutinoside. Total anthocyanins=total amount (sum) of anthocyanins determined by HPLC.

Table 4

Correlation coefficients (*r*) of antioxidant activity with total anthocyanins, total Polyphenols and vitamin C contents

	DPPH	ABTS	FRAP
Total anthocyanins	0.31	0.62	0.16
Total polyphenols	0.77	0.67	0.72
Vitamin C	0.26	0.19	0.02

Table 5

Color parameters of cornelian cherry compote after 1 and 9 months of storage at room temperature

	Fruit with stone (W1)				Fruit without stone (W2)			
	Fruit		Syrup		Fruit		Syrup	
	Means ± SD	Range	Means ± SD	Range	Means ± SD	Range	Means ± SD	Range
				1 month				
*L**	35.53±0.80a	36.53–34.53	44.13±5.01x	35.63–48.17	34.46±0.76a	35.29–33.21	33.24±5.57y	24.55–39.10
*a**	15.09±2.09a	17.35–11.94	64.15±0.98x	63.24–65.51	13.69±2.19a	16.03–10.08	56.33±2.66y	52.76–59.50
*b**	2.49±0.75a	3.03–1.21	70.96±5.90x	61.32–75.81	2.52±0.89a	3.40–1.10	57.15±9.44y	42.32–67.01
Haze	–	–	2.93±1.33y	1.54–4.62	–	–	21.28±3.74x	17.35–25.62
				9 months				
*L**	32.84±1.39a	34.52–31.06	41.49±6.99x	29.59–46.14	32.28±1.06a	33.42–30.81	36.09±5.21x	30.43–44.26
*a**	15.05±2.00a	17.10–12.30	56.18±1.65x	54.86–58.04	16.01±1.96a	18.12–12.81	56.28±2.20x	52.41–57.64
*b**	3.23±0.88a	3.92–1.75	49.57±3.14y	46.76–54.44	4.29±0.94a	5.03–2.69	55.51±2.76x	52.22–58.78
Haze	–	–	2.19±0.51y	1.69–3.02	–	–	13.83±6.94x	7.94–24.82

Mean values within a verses with different letters are significantly different at $P < 0.05$. a,b – for fruit; x, y – for syrup.

Table 5 shows parameters of color of fruits and syrup, in CIE $L^*a^*b^*$ system, after 1 and 9 months of storage. Color parameters after 6 and 9 months were almost the same, therefore only values obtained after 9 months are included in Table 5. After 1 month of storage color parameters L^*, a^*, b^* of syrup were much higher for compote prepared according to variant W1 than W2. Lower differences were found for color parameters of fruits. L^* parameter for syrup ranged from 44 (W1) to 33 (W2), and for fruits – from 35 (W1) to 34 (W2), and during storage its values decreased in most of compotes. The color, both of syrup and of fruits, darkened with the time. Parameter a^* did not change much during storage. For fruits after 1 and 9 months it was, respectively, from 15 to 13 and from 15 to 16. Larger differences in the value of a^* parameter were noted for syrup, where after 1 month it was between 64 and 56, and after 9 months – from 49 to 55. After 1 month of storage, b^* parameter for fruit from compotes W1 and W2 was 2, and for syrup – from 70 (W1) to 57 (W2). During storage the fruits became more, and the syrup less, yellow. For syrup, the measurement of *haze* parameter was performed on apparatus Color –Quest XE. For compotes from fruits without

stone it was noted 7-times more haze than for compotes from fruits with stone. The treatment of stone removal recommended for stone fruits, changed unfavorable the appearance of the fruits, spoiling their structure and causing haze of the syrup. During storage, small parts of fruit flesh, which were the source of haze in compote W2, partially fell to the bottom of jars, and therefore after 9 months decreasing of the haze of syrup was noted.

The results showed that compotes from cornelian cherry fruits were attractive because of their chemical composition, antioxidant activity, and color. Both fruits and syrup are good source of active compounds. Compotes prepared from fruits without stone had, admittedly, worse appearance (larger haze of syrup), but they included larger amounts of stable anthocyanins, vitamin C, acids, and extract. Syrup from cornelian cherry compote, after thinning down to proper extract, may be an alternative to juices and fruit drinks.

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THE MICROBIOLOGICAL QUALITY AND STABILITY OF HIGH PRESSURE PROCESSED CARROT JUICES

Introduction

The health benefits of vegetables are well recognized due to their functional and nutritive properties. The nutritional advantages of vegetables and vegetable products are strictly connected with the high content of saccharides, vitamins, mineral compounds and alimentary fiber. The carrot is rich source of carotenes, other vitamins and mineral compounds with accompanying high dietetic value. According to the physiological studies the appropriation of β-carotene increases with consumption of homogenized vegetable products such the raw carrot juices. The raw carrot juices belongs to perishable products due to undesirable microbiological and enzymatic changes during storage so commercially produced juices are subjected to the process of preservation.

Consumer's expectation for minimally processed, microbiologically safe, addtitives free stable food products with "fresh like" characteristics has stimulated the interest with emerging food technologies. The development of alternative preservation methods has received considerable attention in response to consumer demands for "more fresh and natural" food products [Butz et al., 2003b; Cardello et al., 2007; Deliza et al., 2003; Deliza et al., 2005]. Among non-thermal techniques high pressure processing (HPP) is gaining in popularity not only because of its food preservation capability but also of its potential to achieve interesting functional effects. High pressure processing has potential application for food preservation with prospects to inactivate undesirable microorganisms and enzymes with minimal heat treatment, resulting in the almost complete retention of sensory and nutritional characteristics of fresh food and increased shelf-life [Alpas et al., 2003; Barbosa-Canovas et al., 2005; Bull et al., 2004; Hendrix & Knorr, 2002]. Other advantages of HPP over traditional thermal processing include reduced process time, minimal thermal penetration of product, minimal undesirable functionality alterations, retention of sensory properties as freshness, flavour, texture and colour. Essential nutrients and vitamins undergo minimal or no changes [Butz et al., 2002a, b; Butz et al., 2003a; Houska et al., 2006]. Admission to the usefulness of high pressure treatment of food products is the settlement of such compression circumstances which do not lower the product nutrient values and sensory features and affect directly on his persistence by the elimination or reduction unprofitable enzymatic and microbiological processes [Barbosa-Canovas et al., 2005; IFT Report, 2000; Matser et al., 2004; Torres & Velazquez, 2005].

The vegetative forms of microorganisms according to theirs sensitivity by the impact of high pressures can be divided into three groups: • pressure-sensitive Gram-negative bacteria, subjected to inactivation by minimum pressure value around of 300 MPa, • yeast and

molds, subjected to inactivation by pressures around of 400 MPa, • pressure-resistant Gram-positive bacteria, which inactivation required pressure value of 600 MPa or higher. The most resistant to the effects of high pressures are bacteria spore forms which retaining the ability for growth and reproduction even at pressure of 1000 MPa pressure. Effect of high pressures on microbial morphology depends of the type of microorganisms and environmental conditions of their development (pH, temperature, water activity). The inactivation degree depends of applied high pressure processing parameters and the chemical composition of processed food products. The most effective method of inactivating microorganisms is considered to simultaneous effects of pressure and temperature [Barbosa-Canovas et al., 2005; Farkas & Hoover, 2000; Hendrix & Knorr, 2002, Kim et al., 2001, Ting et al., 2002].

The object of this study was to determine the effect of high pressure processing parameters on microbiological quality and stability of processed carrot juices and to specify the range of microbiological changes in pressurized samples during storage period in refrigeration conditions.

Materials and Methods

The object of study were raw carrot juices extracted from various carrot varieties, recommended for juice production. Raw carrot juices, sealed into sterile, hermetic LDPE type plastic bottles of 50 mL and 100 mL capacity, were pressure processed due to the established compression parameters (pressure/time/temperature) at: 400 MPa/20 min./20^{o}C, 500MPa/10 min./20^{o}C, 500 MPa/20 min./20^{o}C and 600 MPa/10 min./20^{o}C. High pressure processing (HPP) was performed at Institute of High Pressure Researches – UNIPRESS, Polish Academy of Science, Warsaw, Poland, in pilot isostatic pressure chamber (batch type) of appr. 2L capacity. The mixture of distilled water (in 50%) and propylene glycol (in 50%) was used as the pressure medium. The pressure processed samples of carrot juices were analyzed at regular monthly intervals during storage period in refrigeration conditions at 4 ÷ 6^{o}C for up to 3 months. Non-pressurized carrot juices were investigated for comparison of differences to the pressure processed samples. The microbiological analysis of the studied carrot juices were performed according to the PN and PN:ISO standards and were executed in two parallel repetitions. The range of carried out microbiological analysis included the indication level in the presence of: total aerobic mesophilic microorganisms [PN-ISO 4833:1998], coliforms [PN-ISO 4832:1998], lactic bacterial cells [PN-90/A-75052/07], yeasts [PN-ISO 7954:1999], moulds [PN-ISO 7954:1999] and anaerobic spore forms microorganisms [PN-90/A-75052/10] in the studied carrot juices.

Results

The relevant microbiological quality is one of the important factor that determine the food products safety and stability. The quality and durability of preserved juices depends on hygiene and sanitary conditions during the production processes and the effectiveness of the established HPP parameters.

The results of executed microbiological factors analysis in the studied carrot juices are presented in Table 1.

Table 1

Mean values of analyzed microbiological factors in the studied carrot juices [cfu/mL (log cfu/mL)]

Analyzed microbiological factors	HPP parameters [MPa/min./°C]	Studied carrot juices				
		Raw carrot juices	Carrot juices preserved by HPP stored at 4 ÷ 6°C in period [months]			
			0	1	2	3
		[cfu/mL (log cfu/mL)]				
Total aerobic mesophilic microorganisms	400 MPa/20'/20°C	$8,8 \times 10^6$ (6,94)	$2,0 \times 10^6$ (6,30)	$3,2 \times 10^4$ (4,51)	$1,1 \times 10^3$ (3,04)	$9,0 \times 10^0$ (0,95)
	500 MPa/10'/20°C		$2,0 \times 10^6$ (6,30)	$2,0 \times 10^3$ (3,30)	$2,0 \times 10^0$ (0,30)	<1
	500 MPa/20'/20°C	$3,6 \times 10^4$ (4,56)	$1,1 \times 10^2$ (2,04)	$6,0 \times 10^3$ (3,78)	$1,7 \times 10^3$ (3,23)	$2,0 \times 10^0$ (0,30)
	600 MPa/10'/20°C		$2,0 \times 10^0$ (0,30)	$2,0 \times 10^3$ (3,30)	$2,4 \times 10^2$ (2,38)	<1
Bacterial cells of milk fermentation type	400 MPa/20'/20°C	$4,5 \times 10^4$ (4,65)	$2,6 \times 10^4$ (4,41)	$2,3 \times 10^3$ (3,36)	<1	<1
	500 MPa/10'/20°C		$1,8 \times 10^2$ (2,26)	<1	<1	<1
	500 MPa/20'/20°C	<1	<1	<1	<1	<1
	600 MPa/10'/20°C		<1	<1	<1	<1
Coliforms	400 MPa/20'/20°C	$6,6 \times 10^1$ (1,82)	<1	<1	<1	<1
	500 MPa/10'/20°C		<1	<1	<1	<1
	500 MPa/20'/20°C	$2,5 \times 10^2$ (2,40)	<1	<1	<1	<1
	600 MPa/10'/20°C		<1	<1	<1	<1
Moulds	400 MPa/20'/20°C	$1,0 \times 10^0$ (0,00)	<1	<1	<1	<1
	500 MPa/10'/20°C		<1	<1	<1	<1
	500 MPa/20'/20°C	$7,0 \times 10^0$ (0,85)	<1	<1	<1	<1
	600 MPa/10'/20°C		<1	<1	<1	<1
Yeasts	400 MPa/20'/20°C	<1	<1	<1	<1	<1
	500 MPa/10'/20°C		<1	<1	<1	<1
	500 MPa/20'/20°C	<1	<1	<1	<1	<1
	600 MPa/10'/20°C		<1	<1	<1	<1
Anaerobie microorganisms spore forms	400 MPa/20'/20°C	*np	*np	*np	*np	*np
	500 MPa/10'/20°C		*np	*np	*np	*np
	500 MPa/20'/20°C	*np	*np	*np	*np	*np
	600 MPa/10'/20°C		*np	*np	*np	*np

*np – not present

Aerobic mesophilic microorganisms

The aerobic mesophilic microorganisms in the raw carrot juices designed for high pressure processing at 400 MPa/20 min./20°C and 500 MPa/10 min./20°C were present in amount of $8,8 \times 10^6$ cfu/mL (Tab. 1, Fig. 1). The HPP process caused the reduction of analyzed microflora to the identical level of $2,0 \times 10^6$ cfu/mL in the pressurized juices. The researches carried out after the first month of storage period showed the higher

reduction of aerobic mesophilic microorganisms in the samples of carrot juices processed at 500 MPa/10 min./20°C (to $2,0 \times 10^3$ cfu/mL) than in the juices processed at 400 MPa/20 min./20°C (to $3,2 \times 10^4$ cfu/mL). The similar tendency of changes was noticed after the second month of storage period, where the analyzed microbiological factor decreased in greater extent in the carrot juices pressurized at 500 MPa/10 min./20°C (by 3 logarithmic cycles, to the level of $2,0 \times 10^0$ cfu/mL) in comparison with the juices processed at lower pressure: 400 MPa/20 min./20°C (by 1 logarithmic cycle, to the level of $1,1 \times 10^3$ cfu/mL). Based on the researches carried out after the third month of storage period the presence of investigated microflora was not detected in the samples of carrot juices pressurized at 500 MPa/10 min./20°C while in the juices samples processed at 400 MPa/20 min./20°C was present in amount of $9,0 \times 10^0$ cfu/mL (decreased in three logarithmic cycles) (Tab. 1, Fig. 1).

The range of changes in amount of aerobic mesophilic microorganisms present in the carrot juices processed at 400 MPa/20 min./20°C illustrates the trend line of second-degree multinomial type with the value of multiple correlation coefficient $R^2 = 0,9953$ as the equation $y = -0,1843x^2 - 0,4183x + 7,63$. The trend line of second-degree multinomial type with the value of multiple correlation coefficient $R^2 = 0,9392$ as the equation $y = 0,0486x^2 – 2,2794x + 9,672$ represents the range of changes in number of aerobic mesophilic microorganisms present in the studied carrot juices processed at 500 MPa/10 min./20°C (Fig. 1).

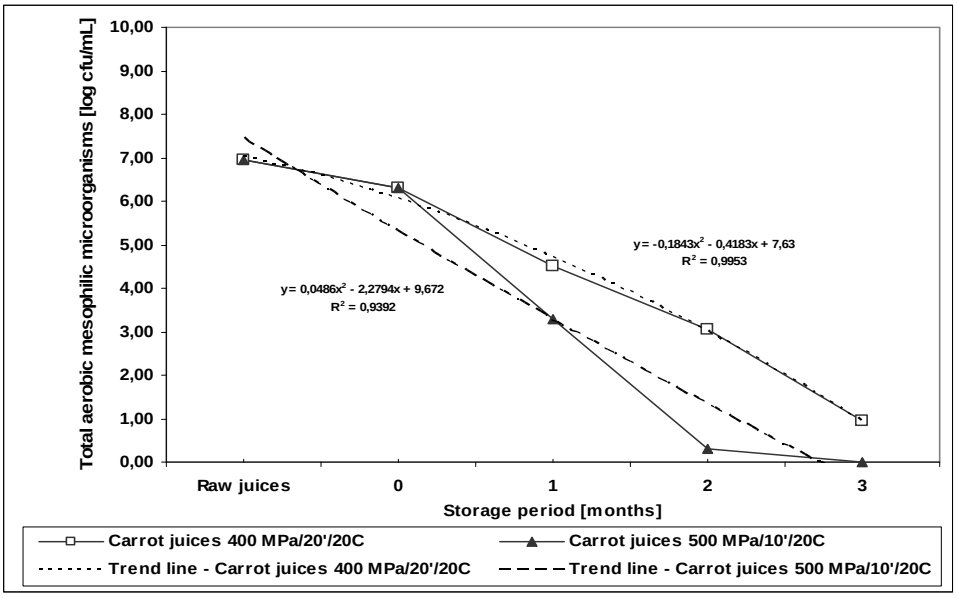

Fig. 1. The range of changes in presence of total aerobic mesophilic microorganisms in the studied carrot juices processed at 400 MPa/20 min./20°C and 500 MPa/10 min./20°C [log cfu/mL]

In the raw carrot juices designed for high pressure processing with compression parameters: 500 MPa/20 min./20°C and 600 MPa/10 min./20°C the aerobic mesophilic microorganisms were present in amount of $3,6 \times 10^4$ cfu/mL (Tab. 1, Fig. 2). The analysis executed directly after pressurization process showed high degree reduction of studied microflora in

the carrot juices processed at 600 MPa/10 min./20°C (by 4 logarithmic cycles to $2,0 \times 10^0$ cfu/mL) while in the juices processed at 500 MPa/20 min./20°C the amount of aerobic mesophilic microorganisms decreased to $1,1 \times 10^2$ cfu/mL. After the first month of storage period an increase of investigated mesophiles was found with higher degree in the juices pressurized at 500 MPa/20 min./20°C (to the level of $6,0 \times 10^3$ cfu/mL) than in processed juices at 600 MPa/10 min./20°C (to $2,0 \times 10^3$ cfu/mL). The analysis carried out after second month of storage period showed the higher degree of inactivation of aerobic mesophiles in the pressurized carrot juices processed at 600 MPa/10 min./20°C (to $2,4 \times 10^2$ cfu/mL) than at 500 MPa/20 min./20°C (to $1,7 \times 10^3$ cfu/mL). Based on the researches carried out after the third month of storage period the presence of investigated microflora was not detected in the samples of carrot juices pressurized at 600 MPa/10 min./20°C while in the juices samples processed at 500 MPa/20 min./20°C the amount of mesophiles decreased to $2,0 \times 10^0$ cfu/mL (Tab. 1, Fig. 2).

The range of changes in amount of studied mesophiles present in the carrot juices pressurized at 500 MPa/20 min./20°C illustrates the trend line of third-degree multinomial type with the value of multiple correlation coefficient $R^2 = 0,9461$ as the equation:
$y = -0,5533x^3 + 4,7579x^2 - 12,459x + 12,722$. The trend line of the third-degree multinomial type with the value of multiple correlation coefficient $R^2 = 0,8254$ as the equation:
$y = -0,7267x^3 + 6,5286x^2 - 17,785x + 16,348$ represents the range of changes in number of aerobic mesophilic microorganisms present in the juices processed at 600 MPa/10 min./20°C (Fig. 2).

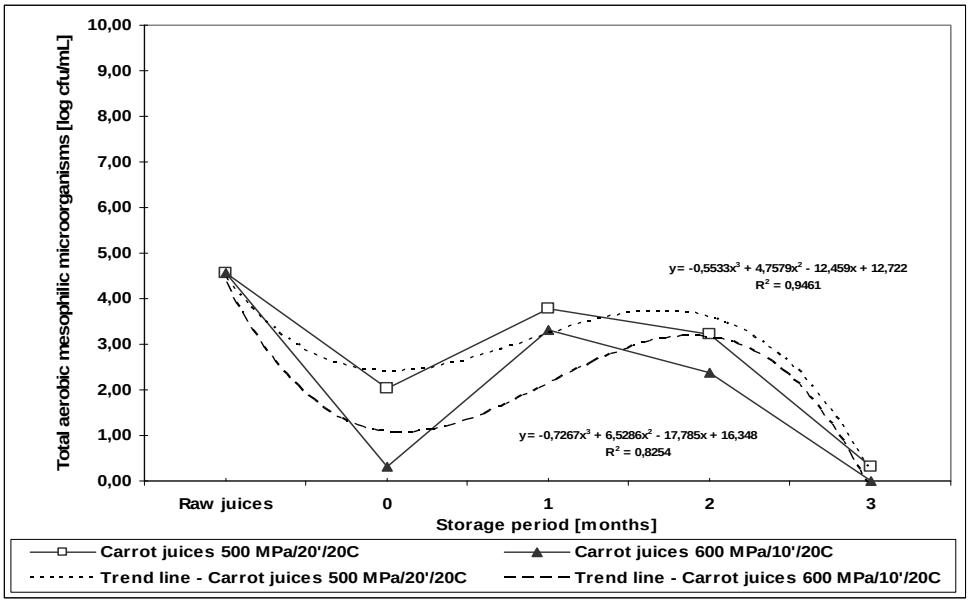

Fig. 2. The range of changes in presence of total aerobic mesophilic microorganisms in the studied carrot juices processed at 500 MPa/20 min./20°C and 600 MPa/10 min./20°C [log cfu/mL]

Lactic bacterial cells

The lactic bacterial cells in the raw carrot juices designed for high pressure processing at 400 MPa/20 min./20°C and 500 MPa/10 min./20°C were present in amount of $4,5 \times 10^6$ cfu/mL. (Tab. 1, Fig. 3). The microbiological analysis carried out immediately after presurrization process showed higher degree of inactivation of studied microflora in the juices processed at 400 MPa/20 min./20°C (by 4 logarithmic cycles, to $1,8 \times 10^2$ cfu/mL) than in the juices processed at 500 MPa/10 min./20°C (by 2 logarithmic cycles, to $2,6 \times 10^4$ cfu/mL). The presence of lactic bacterial cells was not detected after the first month of storage period in the carrot juices pressurized at 500 MPa/10 min./20°C while in the juices pressurized at 400 MPa/20 min./20°C the studied microflora was reduced to amount of $2,3 \times 10^3$ cfu/mL. The microbiological analysis performed after the second and the third month of storage period have not shown the presence of lactic bacterial cells in the studied carrot juices (Tab. 1, Fig. 3).

The trend line of the third-degree multinomial type with the value of multiple correlation coefficient $R^2 = 0,9659$ as the equation $y = 0,3475x^3 - 3,2582x^2 + 7,6143x - 0,156$ represents the range of changes in amount of lactic bacterial cells present in the juices processed at 400 MPa/20 min./20°C. The range of changes in amount of studied lactic bacterial cells present in the carrot juices pressurized at 500 MPa/20 min./20°C illustrates the trend line of third-degree multinomial type with the value of multiple correlation coefficient $R^2 = 0,984$ as the equation $y = -0,0108x^3 + 0,6004x^2 - 4,4288x + 8,522$ (Fig. 3).

The executed microbiological analysis did not reveal the presence of lactic bacterial cells in the raw carrot juices and carrot juices processed with scheduled HPP parameters: 500 MPa/20 min./20°C and 600 MPa/10 min./20°C during the entire cycle of study (Tab. 1).

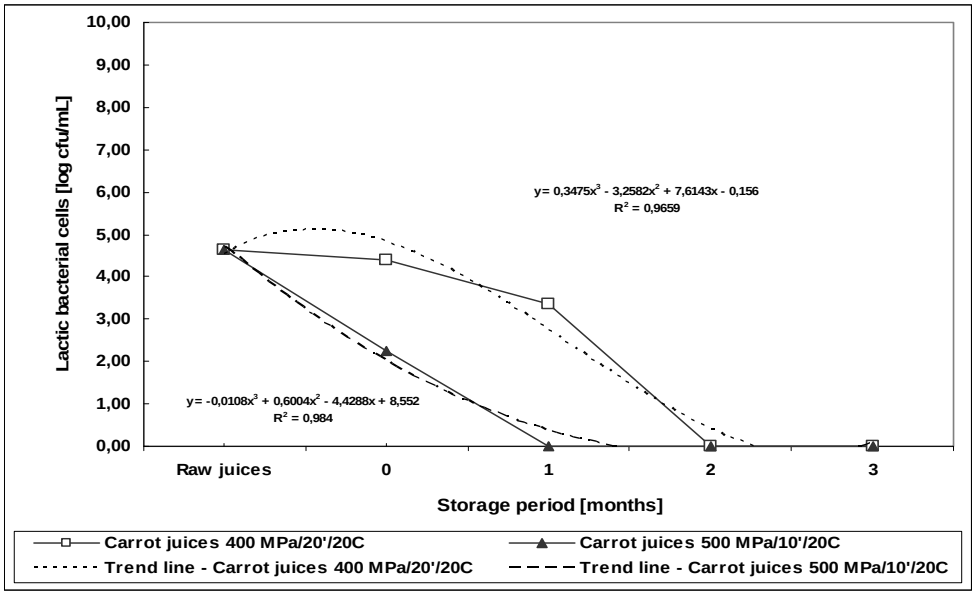

Fig. 3. The range of changes in presence of lactic bacterial cells in the pressurized carrot juices processed at 400 MPa/20'/20°C and 500 MPa/10'/20°C [log cfu/mL]

Coliforms

The presence of coliforms in the raw carrot juices designed for pressurization at 400 MPa/20 min./20°C and 500 MPa/20 min./20°C was found in amount of $6{,}6 \times 10^1$ cfu/mL. The carrot juices intended for high pressure processing at 500 MPa/20 min./20°C and 600 MPa/10 min./20°C contained the coliforms in amount of $2{,}5 \times 10^2$ cfu/mL. The executed microbiological analysis executed immediately after pressurization process and during storage period of the processed carrot juices did not reveal the coliforms presence (Tab. 1). Coliforms belongs to the pressure sensitive Gram-negative bacteria and subjected to inactivation after the application of high pressures around 300 MPa. The impact of high pressure causes damage of cell membranes leading to destruction of intracellular bacterial structures.

Moulds

The raw juices of carrots intended for high pressure processing at 400 MPa/20 min./20°C and 500 MPa/20 min./20°C contained moulds in amount of $1{,}0 \times 10^0$ cfu/mL. The presence of moulds in the raw carrot juices designed for high pressure processing at 500 MPa/20 min./20°C and 600 MPa/10 min./20°C 400 MPa/20 min./20°C was found in amount of $7{,}0 \times 10^0$ cfu/mL. The microbiological analysis carried out directly after pressurization process and during storage period of the processed juices have not shown the moulds presence (Tab. 1). Moulds as well as yeasts are subjected to inactivation after the application of high pressures values of 400 MPa.

Yeasts

Based on the carried out microbiological analysis no presence of yeast was found in the raw carrot juices and the pressure processed juices at 400 MPa/20 min./20°C and 500 MPa/20 min./20°C, 500 MPa/20 min./20°C and 600 MPa/10 min./20°C, during the entire cycle of performed studies (Tab. 1).

Anaerobic spore forms microorganisms

The carried out microbiological analysis did not reveal the presence of anaerobic spore forms microorganisms spore forms in the raw carrot juices and the pressurized carrot juices processed at 400 MPa/20 min./20°C and 500 MPa/20 min./20°C, 500 MPa/20 min./20°C and 600 MPa/10 min./20°C during the entire cycle of performed studies (Tab. 1).

Conclusions

The results of microbiological analysis indicated the positive effect of high pressure processing on quality and stability of processed and stored carrot juices which presented acceptable sensory properties up to the first month of storage period. The pressure treatment of carrot juices reduced the microbial load to non-detectable level or significantly reduced the population of studied microorganisms. The inactivation level of the analyzed microorganisms was more effective in the samples processed at higher pressure values: 500 MPa and 600 MPa. Quality changes having influence on stability of the pressurized carrot juices could be attributed to possible microorganisms regeneration after high pressure processing and requires to apply the appropriate high pressure values with mild temperature treatment as compression parameters to improve product safety and stability.

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APPLICATION OF ULTRASOUNDS FOR ELIMINATION OF SELECTED GRAM-POSITIVE PATHOGENIC BACTERIA CONTAMINATING POULTRY CARCASSES

Introduction

Ultrasounds are the object of intensive research in food industry, because they hale wide variety of applications in the processing and evaluation of products. Among them ultrasound is able to inactivate bacteria and deagglomerate bacterial clusters or flocks through a number of physical, mechanical and chemical effects arising from acoustic cavitation. On collapse, cavitation bubbles produce enough energy to mechanically weaken or disrupt bacteria via number of processes [Joyce et al., 2003, Knorr et al., 2004, Salleh-Mack and Roberts, 2007]. The mechanism of microbial killing is mainly due to thinning of cell membranes, localized heating and free radicals producing. During sonication, longitudinal waves are created when a sonic wave meets a liquid medium, thereby creating the regions of alternating compression and expansion [Piyasena et al., 2003]. Synergistic effects of ultrasound with other agents such as temperature, high pressure, lactic acid, TiO_2 particles have been also examined [Piyasena et al., 2003, Ross et al., 2003, Stasiak et al., 2007, Rodriguez-Calleja et al., 2006, Drakopoulou et al., 2009].

Poultry meat production worldwide has increased rapidly with annual growth rate of 6% [Bolder, 1998]. This has led to intensive animal production, which favors the hygienic quality of the final product. Live poultry being normally raised on litter floors, contains a lot of microorganisms e.g. pathogens such as *Salmonella, Campylobacter, Listeria*, *E. coli* and *S. aureus*. Microorganisms that are isolated from fresh carcasses come from feathers, skin, intestinal tract and processing lines [Bolder, 1998]. It was confirmed, that low microbiological quality of poultry products in Poland was on the level 3,3% of the surveyed samples [Statistical Yearbook, 2008].

The heterogeneous microflora on processed poultry consists of both pathogenic and spoilage microorganisms: *Salmonella, Campylobacter*, that are the major cause of human infections. On the other hand, isolation rate of *S. aureus, E. coli, Listeria, Aeromonas*, *Clostridium* increases all the time. There is little information about pathogens such as *Yersinia, Hafnia, Bacillus* and others [Bolder, 1998].

During poultry processing, contamination levels should be controlled by taking hygienic measures, based on HACCP principle but complete eradication of pathogens seems impossible without additional decontamination treatment [Bolder, 1998].

The purpose of the study was to investigate ultrasounds effect on two pathogenic, Gram-positive bacteria: *Staphylococcus aureus* and *Bacillus cereus* that cause some gastrointestinal disturbances. Because there is a drive towards the use of sonication as an adjunct to other techniques, simultaneous effect of ultrasound and lactic acid was examined.

Materials and methods

Materials

The chicken wings were obtained from the Indykpol poultry slaughterhouse (Lublin, Poland). Gram-positive bacteria: *Staphylococcus aureus* and *Bacillus cereus* were taken from collection of Department of Biotechnology, Human Nutrition and Food Commodities of University of Life Sciences in Lublin. Strains were stored on agar slants in +4ºC and occasionally inoculated.

Sonication treatment

Beginning experiment controlled the reaction of investigated bacteria on sonication conditions and working aliquot. Bacteria were sonicated for 5, 10, 15 min in water or 0,8% aqueous solution of lactic acid . The samples of 1 cm^3 were taken for microbiological analysis. Sonication was realized with bath device (Polsonic) under the following conditions: frequency 40 kHz and intensity 2 W/cm^2.

After selection of sonication environment, chicken wings were investigated. They were challenged with *S. aureus* or *B. cereus* by plunging in bacteria suspension containing 2,5 x 10^6 – 8,8 x 10^7 cfu/cm^3 of cocci and 1,8 x 10^6 – 4,1 x 10^8 cfu/cm^3 of bacilli for 15 min. The infected wings were packed in sterilized aluminum foil and lost for 30 min in the aim of better adsorption of bacteria to the skin. After this time wings were put into ultrasonic bath and sonicated. Ultrasound treatment was conducted in 0,8, 2,4 and 4,0% lactic acid aqueous solutions. Three periods of sonication: 5, 10 and 15 min were used. One infected wing was used as unsonicated control sample. After appropriate time of sonication, samples were put out and two smears from 4 cm^2 of surface were taken from one wing for the microbiological analysis. Smears were also collected from control wing. The 1 cm^3 of aliquots were taken from sonic bath after sonication and decimal dilutions were prepared to determine the presence of living bacteria in reaction environment. Experiments were duplicated.

Lactic acid treatment

Simultaneously, control experiments on chicken wings were done using only lactic acid aqueous solutions in concentrations 0,8, 2,4 and 4%, in which samples were plunged and stored for 5, 10 and 15 min without sonication. The aim of these kind of control was to study the bactericidal effect of lactic acid alone on staphylococci and bacilli and assessment of synergistic effectiveness of both agents used. The whole experiment was done according to the method described above without sonication stage.

Microbiological analysis

From each sample (aliquot and smears) decimal dilutions in sterile physiological salt solution were prepared and 1 cm^3 of each dilution was put and spread on sterile Petri plates. Baird-Parker and MYP (acc. Mossel) (BTL) agars were used for growth of *S. aureus* and *B. cereus*, respectively. Incubation at 37°C for 24–48 h was continued. Characteristic colonies were counted. *S. aureus* colonies on Baird-Parker agar are small, round, black, shiny with transparent zone around. Colonies of *B. cereus* on MYP agar are big, rough, flat and pink.

The numbers of colonies forming units (cfu) in 1 cm^3 of aliquots or in 1 cm^2 of chicken wings surface are presented as logarithms from cfus. The results are presented as mean of two independent experiments, with standard deviation.

Results and discussion

In the first experiment, it was observed that both *S. aureus* and *B. cereus* were more sensitive on ultrasound in lactic acid aqueous solutions (0,8 and 2,4% v/v) than in water, although its reduction was low (Fig. 1). The results obtained suggested that to get better bactericidal effect, the solution of lactic acid should be used as sonication environment. The positive influence of acidity on ultrasound effectiveness was also observed by Salleh-Mack and Roberts [2007], who investigated the bactericidal effect of sonication used with different agents (organic acids, pH, soluble solids) on *E. coli* ATCC 25922 reduction [Salleh-Mack and Roberts, 2007].

Ultrasound treatment of wings infected with *S. aureus* in 0,8% (v/v) lactic acid solution was only partially effective in elimination of microorganism and it was not depended on the process time. The reduction of bacteria count on the surface was about 1,5 log at the beginning contamination amounted to 3,9 log cfu/cm^2. Simultaneously, there were observed living bacteria in the working aliquot in the number between 1,78 and 3,68 log cfu/cm^3 for different series and sonication time. The most bactericidal effect on *S. aureus* was obtained in 2,4% (v/v) lactic acid during 15 min of sonication. Reduction of cocci of 3,57 logarithmic cycles from cfu/cm^2 was obtained what was totally reduction of living cocci on the surface. They were also absent in lactic acid solution. During sonication period of 5 and 10 min in 2,4% (v/v) lactic acid solution reduction on the level 2,6–2,8 log cfu/cm^2 was observed, but living cells on the surface of wing were detected. However they were absent in working aliquot (Fig. 2 and 3). Comparing with the action of lactic acid alone, better effect was obtained, reduction of bacteria count was higher on 1,76–2,2 logarithmic cycle depending on sonication time. Application of 4% aqueous solution of lactic acid was also effective, reduction of bacterial cells on wings was satisfying and cells were no detectable in aliquot. The reduction of bacteria count was higher on 0,22–1,36 logarithmic cycle in comparison with the action of lactic acid alone and depended on sonication time. Obtained results are presented at Fig. 2 and 3. Rodriguez-Calleja et al. [2006] investigated the influence of sonication with pressure (manosonication) on different food isolates of *S. aureus* and stated that decimal reduction times ranged from 5,3 to 1,3 min and these values were greater than those reported for most vegetative species investigated under similar conditions [Rodriguez-Calleja et al., 2006]. Stasiak et al. [2007] concluded that longer sonication time was more effective in reduction of microorganisms count. They observed that total count of bacteria on wings skin decreased more than 1,8 log after 6 min of sonication in water and shorter exposition time gave weaker effect of decontamination: 1 min – 0,2 log and 3 min – 1,2 log. On the other hand they showed that water was better working solution than 1% (v/v) aqueous solution of lactic acid for reduction of total count of microorganisms. Simultaneously living cells were observed in liquid, probably from the phenomenon of washing of bacteria from the skin [Stasiak et al., 2007].

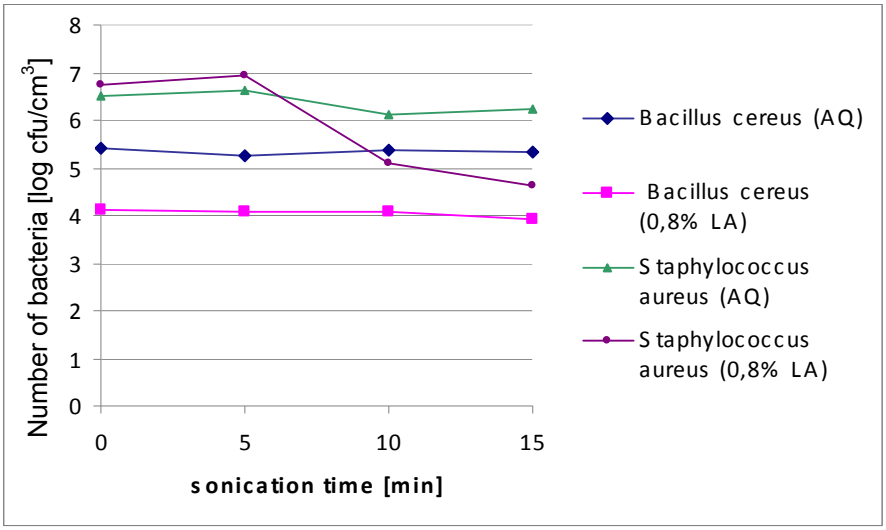

Fig. 1. The influence of sonication time on growth of *S. aureus* and *B. cereus* in water (AQ) or 0,8% aqueous solution of lactic acid (LA)

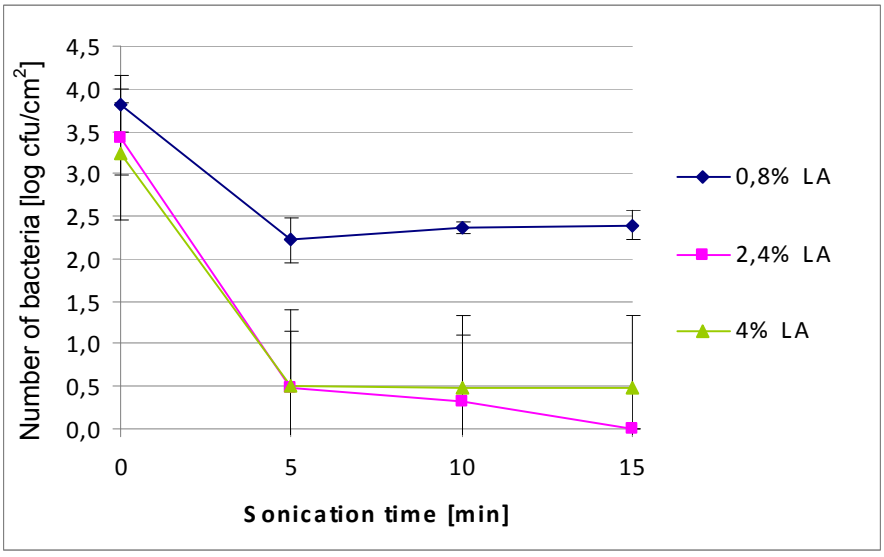

Fig. 2. The influence of sonication time and lactic acid aqueous solution (LA) on number of *S. aureus* on wing's surface

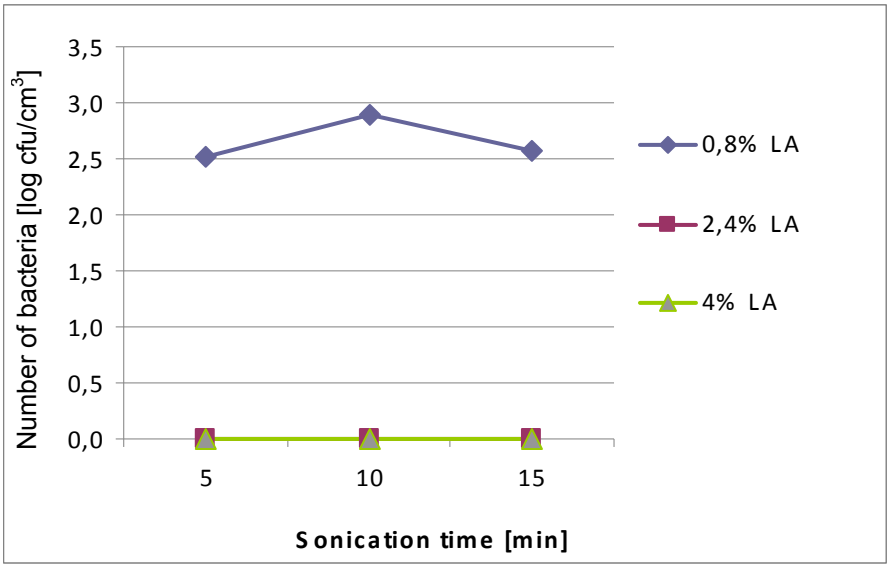

Fig.3. The number of living bacteria *S. aureus* in lactic acid solutions (LA) as working aliquot

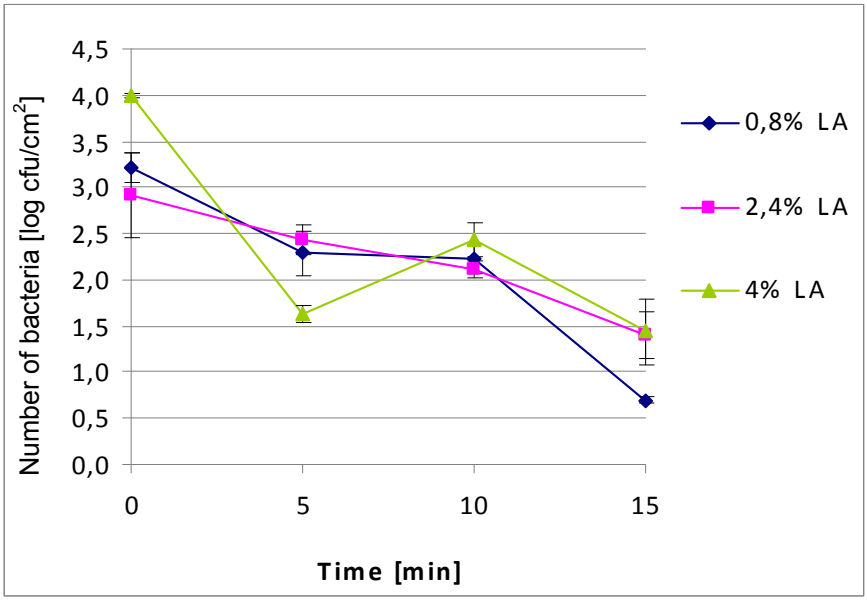

Fig. 4. The influence of lactic acid solutions' treatment (LA) on number of *S. aureus* on wing's surface

Sonication in 0,8% (v/v) lactic acid solution for 10 min or in 2,4% (v/v) acid for 5 min was effective in total elimination of *B. cereus* cells from chicken wings and also from working aliquot (Fig. 5 and 6). However 5 min of sonication in 0,8% (v/v) lactic acid solution gave reduction of bacilli count on the wing surface at the mean level about 2 logarithmic cycle on cm^2. Shortening of sonication time of infected *B. cereus* wings to 1 or 3 min was also partially effective, the count of living cells on the surface decreased about 2 log cfu/cm^2, what was successful. The number of bacteria declined on 98,7–99,2%. but active bacteria were observed in lactic acid solution after sonication for 5 min (Fig. 6). However Joyce et al. [2003] indicated that at low frequency ultrasound (20 and 38 kHz) there was no dramatic effect on the viability of *B. subtilis* cells in nutrient broth, but at 15 min of sonication this effect was detectable. In contrast the higher frequency produced the rise in the cfu followed by steady fall that suggested that the major effect of such kind of ultrasound made declumping of bacterial agglomerates with little deactivation. They also observed that bioeffect of sonication was depended on sample volume and was higher for smaller volumes, where the sound intensity was higher [Joyce, 2003].

Synergistic activity of both used agents that was observed in the present experiment was more effective than action of lactic acid. (Fig. 4 and 7). It has been shown that *S. aureus* was more resistant on the low frequency and medium intensity ultrasound than vegetative cells of *B. cereus*. Moreover, the time of sonication was important. According to Scherba et al. [1991] bacteria such as *S. aureus, B. subtilis, P. aeruginosa* were affected by the ultrasound with the bactericidal effect increasing with the time and intensity. The reduction of *B. subtilis* ranged from 52 to 76% and from 42 to 43% for *S. aureus* when sonication time was increased from 2 to 30 min at the ultrasound power intensity of 3 W/cm^2. The reduction of microorganisms increased from 11 to 100% for *B. subtilis* and from 22 to 39% for *S. aureus* when treatment intensity was increased from 1 to 3 W/cm^2 after 15 min of sonication [Piyasena et al., 2003]. Villamiel and de Jong [2000] reported that Gram-negative bacteria (*Pseudomonas fluorescens*) were more susceptible to the ultrasonic treatment than Gram-positive (*Streptococcus thermophilus*). This observation was in agreement with the present work and our previous observations [Stasiak et al., 2007] and with Hulsen [1999] and Alliger [1975] who reported that Gram-negative rod shaped bacteria were more sensitive than Gram-positive coccus shaped bacteria [Villamiel and de Jong, 2000]. Similar conclusions were drawn by Drakopoulou et al. [2009], who studied the influence of ultrasound (24 kHz) with or without particles of TiO_2 on different bacteria present in municipal wastewater such as coliforms, *Pseudomonas* spp., enterococci and *Cl. perfringens* [Drakopoulou et al., 2009]. They obtained higher reduction of Gram-negative bacteria (99,9%) than Gram-positive strains (72,8–87,1%) using sonication with TiO_2 for 60 min In contrast, Scherba et al. [1991] found no differences in resistance to ultrasound between Gram-negative and Gram-positive such as *S. aureus* and *B. subtilis*. They argued that differences in cell wall structure did not seem to be a differentiating factor in ranking of agents influencing ultrasonic efficiency [Piyasena et al., 2003]. The various parameters of sonication treatment are used in the scientific laboratories and this fact differentiates the research results. The main method of optimization of microbial inactivation by ultrasound is the laboratory experiment in properly described conditions.

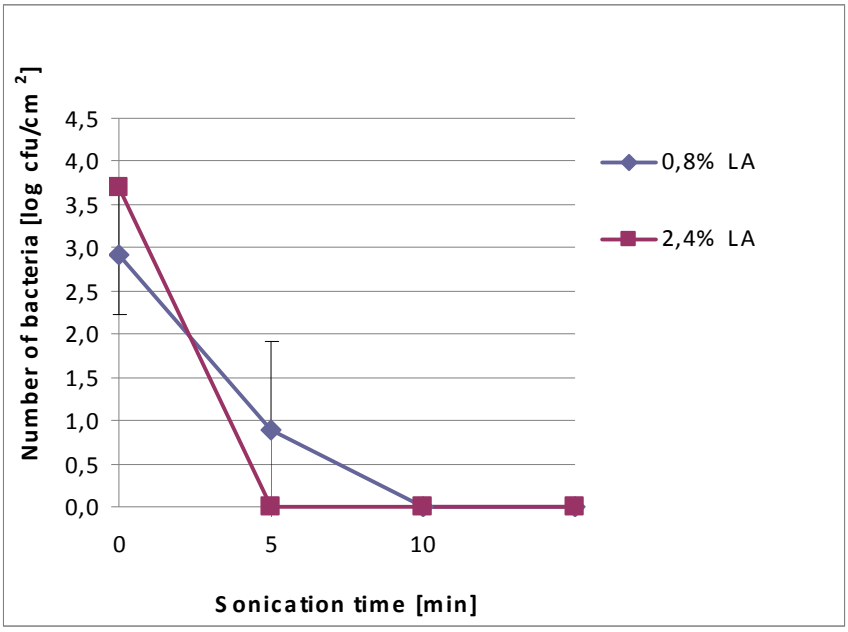

Fig. 5. The influence of sonication time and lactic acid aqueous solution (LA) on number of *B. cereus* on wing's surface

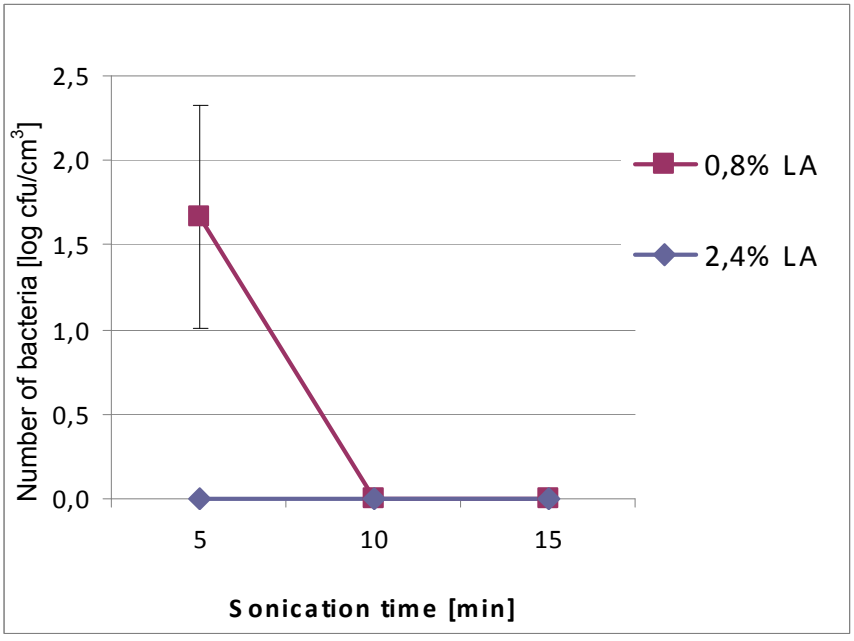

Fig. 6. The number of living bacteria *B. cereus* in lactic acid solutions (LA) as working aliquot

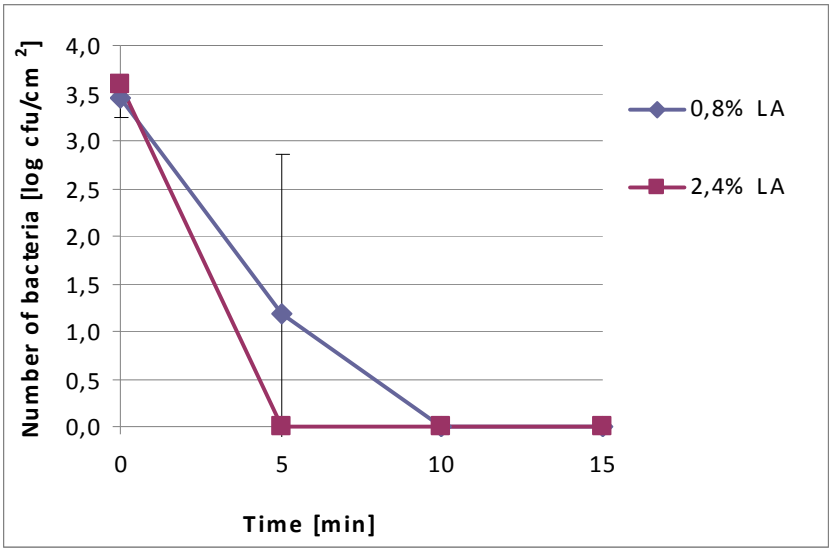

Fig. 7. The influence of lactic acid solutions' treatment (LA) on number of *B. cereus* on wing's surface

Conclusions

The performed studies confirmed possibility of ultrasound application for reduction or elimination of Gram-positive bacteria such as *S. aureus* or *B. cereus*, that can contaminate poultry carcasses and cause gastrointestinal efforts. There were better results obtained for simultaneous action of sonication and lactic acid aqueous solutions (0,8, 2,4 or 4% v/v) as working aliquots. Obtained results showed that *S. aureus* was more resistant on used agents than *B. cereus* and for elimination of cocci from wings' skin the sonication for 15 min in 2,4% lactic acid solution was effective while for bacilli this process in 0,8% lactic acid solution for 10 min was sufficient. Alive bacteria were absent also in working aliquots what confirmed bactericidal effect but not only washing effect of used agents. Presented results of this experiment are generally in agreement with similar investigations. They confirm utility of ultrasound together with lactic acid (acid environment) for decontamination of food. Obviously, there is a need for further studies on ultrasound action on microorganisms and application of this method in practice.

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UV-C IRRADIATION SANITATION OF SHELL SURFACE OF EGGS LAID BY LAYER HENS IN LITTER HOUSING

Introduction

Eggs are an important component in human diet due to their nutritive value [Surai et al., 2001]. Eggs directly after being laid over their entire shell surface contain from 10^4 to 10^7 bacterial cells. However, their count increases with the degree of shell soiling. The degree of egg shell soiling is affected, among other things, by the housing management system for layer hens. It was shown that the shell surface of eggs from the litter housing system is over 15 times more contaminated than eggs from the cage housing system. Bacterial contamination pertains to many types of bacteria, e.g. *coliform*, among which there may be pathogenic serotypes of *Escherichia coli* and relatively often *Salmonella* [Musgrove et al., 2008]. Both bacteria are frequently etiological factors of diarrheas.

In recent years the number of infections and food poisonings has been increasing worldwide, among which salmonelloses and colibacterioses predominate. It is eggs and processed egg products that are perceived as the most frequent sources of these bacteria.

Pathogenicity of *E. coli* depends on its invasiveness and potential to produce toxins. Invasive strains of these bacteria have the ability to penetrate tissues and cause inflammatory reactions, thus they cause inflammations of the alimentary tract and septicaemias. Apart from toxin-forming strains producing large amounts of toxins in the intestines, which leads to enterotoxicaemia, there are strains with invasive and toxigenic properties, capable of causing both the so-called gastroenteritis and enterotoxicaemia [Braun et al., 2005]. Sensitivity of *E. coli* to environmental factors, including disinfectants, is relatively high. Inactivation occurs after 20 min heating at 60°C. However, in a medium with a lower temperature and adequate humidity they may retain viability for even several months [Chavez et al., 2002]. The most dangerous strain of genus *E. coli* transferred also through food is an enterohemolytic strain of bacteria *E. coli* O157:H7 causing diseases of the alimentary and urinary systems. It produces verocytotoxin, a substance causing diarhheas and complications in diseases of adrenal glands and a dangerous haemolytic-uraemic syndrome, usually fatal. The primary cause of infection is the consumption of food not subjected to thermal processing [Chavez et al., 2002]. The intensity and course of disease vary considerably and are dependent on individual sensitivity and the amount of bacteria, which entered the body [Tosa et al., 1999].

The scale and gravity of the problem of bacterial contamination of laying houses with pathogenic bacteria are reported by the European Food Safety Agency (EFSA). In June 2006 it published a report indicating that over 55% laying houses in commercial flocks of layer hens are contaminated with Gram-negative bacteria. In relation with this the European

Commission, on the power of Ordinance no. 1168/2006 of 31 July 2006, among other things imposed on Poland the obligation to reduce the level of bacteria from genus *Salmonella* found with high frequency in poultry. This obligation concerns the reduction by the end of 2010 of the incidence of *Salmonella* to 1% in commercial flocks of layer hens.

One of the recommended methods to reduce the level of microbiological contamination on the shell surface of eggs for human consumption is UV-C irradiation at 254 nm. The sanitation effect could be better if it was applied after shell surface had been washed [Hutchinson et al., 2005]. This procedure aims at a reduction of bacterial counts on egg shells, which improves their hygienic quality and which has been applied successfully for many years in Sweden, the USA, Canada and Japan to prepare eggs for human consumption to retail sale [Braun et al., 2005].

The range of wave lengths in UV radiation is from 100 to 400 nm, neighbouring with X radiation and the spectrum of visible light radiation.

In terms of their specific properties we distinguish:

a) the far range 100 to 280 nm (UV-C), characterized by bactericidal properties, with a maximum action at 254 nm,
b) the medium range 280 to 315 nm (UV-B), catalyzing chemical and biochemical reactions,
c) the close range 315 to 400 nm (UV-A), activating object luminescence [Bintsis et al., 2000].

In practice we use the UV-C range and wave length of 254 nm, which causes an immediate photochemical reaction in DNA, initiating its mutations. Different doses of irradiation are required to inactivate different microorganisms, with these doses for a given microorganism depending on the fact whether they include its photoreaction. It was shown that bacteria not producing spores are more easily destroyed than spore-producing bacteria, while the highest resistance to inactivation by UV-C is found for fungi and yeasts. The effectiveness of ultraviolet radiation depends e.g. on the development phase of microorganisms, the development and irradiation medium as well as cell counts. Air temperature ranging from 5 to 37°C does not have a significant effect on the bactericidal action of ultraviolet radiation [De Reu et al., 2006].

UV-C radiation at 254 nm is characterized by poor radiation hardness in opaque media. Thus in case of foodstuffs it may be used only for surface sterilization. The other limitation in the application of UV radiation results from its strong absorption by particles of dust and moisture layers, as well as the effect of their action rapidly changing with an increase in the distance from the source of radiation [Bintsis et al., 2000]. Our earlier studies showed a lack of negative effects of action of UV-C radiation on egg content. Moreover, a considerable effectiveness was observed in the inactivation of pathogenic bacteria *E. coli* PCM 843 and *S. enteritidis* PCM 413 inoculated on the surface of egg shells [Szablewski et al., 2009].

The aim of the study was to evaluate the effect of shell sanitation by UV-C irradiation in eggs laid by layer hens in the litter housing system, in combination with earlier washing of shell surface, by determining changes in native bacterial microflora on the shell surface.

Material and methods

Eggs of layer hens were irradiated with UV-C radiation at 254 nm in an egg irradiator UV 254 by CompArt. A total of 696 eggs were analyzed in the experiment, of which 232 samples were prepared for microbiological analyses.

Analyses were conducted directly after irradiation of egg surface and after 2 and 4 weeks of their cold storage in relation to controls – non-irradiated. Different irradiation times of 30, 60 and 90 s were applied. The experiment was conducted using eggs with soiled shell surface and with washed shells – eggs with a soiled shell surface were washed under running water at 18–20°C to obtain a visually clean shell surface.

Irradiated and control eggs were broken onto Petri dishes and shells were placed in sterile bags. A single sample for microbiological assays consisted of shells from 3 eggs. Shells were crushed and 10 g were transferred to a flask with 90 ml buffered peptone water. Thus prepared samples were shaken for 15 min. in order to remove bacteria from shell pores. From the obtained 10^{-1} dilution successive decimal dilutions were produced and inoculated using the classical plate flooding method according to Koch towards the total microbial count and the count of coliform bacteria. Total bacterial counts were determined using agar medium (BTL), while coliform bacteria - using a ChromAgar ECC medium (Graso). Samples were incubated at 30°C for 72 h and 37°C for 24 h, respectively. Calculations were conducted for dilutions from plates, on which the number of colonies ranged from 30 to 300. Results were expressed in CFU/g shell. For the analysis of means descriptive statistics were used together with 95% confidence intervals. Moreover, the HSD Tukey`s test was applied at the level of significance $\alpha = 0.05$.

Results and discussion

Statistical analysis showed statistically significant differences between total microbial counts on the surface of egg shells irradiated with different doses of radiation, expressed in irradiation times.

In case of eggs with soiled surface the initial level of total mesophilic microflora was 6.8 log, while after irradiation with UV-C for 90 s it decreased to 5.8 log. For eggs with washed surface this reduction was from 4.4 log to 3.4 log. Irrespective of irradiation time a retained reduction was observed in the total bacterial counts between the surface of washed eggs and eggs with soiled shell surface by over 2 log (Fig. 1).

In case of coliform bacteria statistically significant differences were found only in case of the effect of irradiation time for eggs with washed surface. Reduction of bacterial counts was from 2.9 log to 1.5 log (Fig. 2).

De Reu et al. [2006] investigated changes in total bacterial counts on the shell surface of visually clean and soiled eggs under the influence of UV-C radiation. Eggs were irradiated using a mobile conveyor with two belt speeds: 10 000 eggs/h, and 2 500 eggs/h, for 4 and 18 s. These studies confirmed the effectiveness of radiation in case of the surface of visually clean eggs, where microbial counts decreased by 1 log, and a lack of efficiency in case of the surface of soiled eggs.

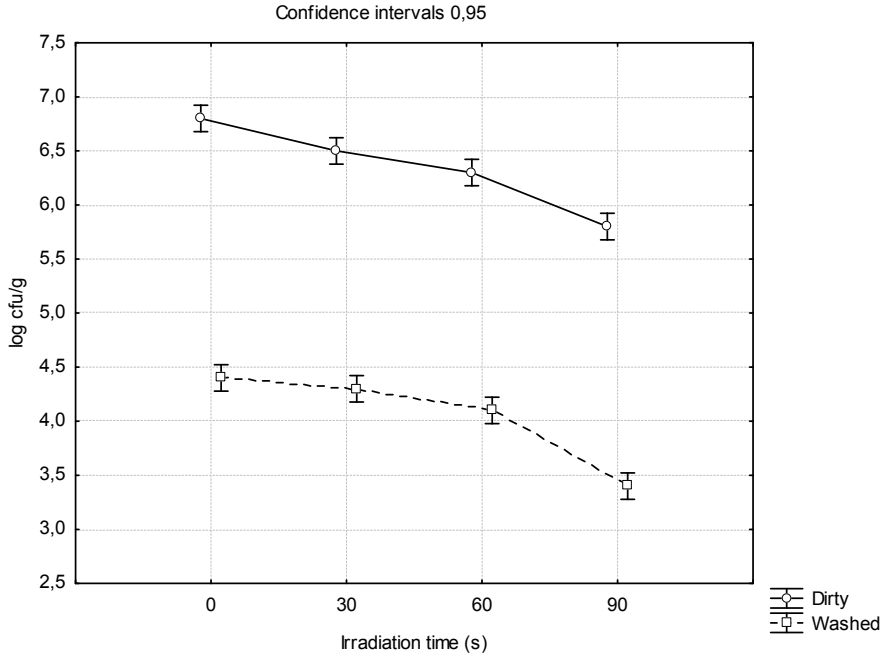

Fig. 1. The effect of UV-C irradiation time on total bacterial count per 1 gram shell

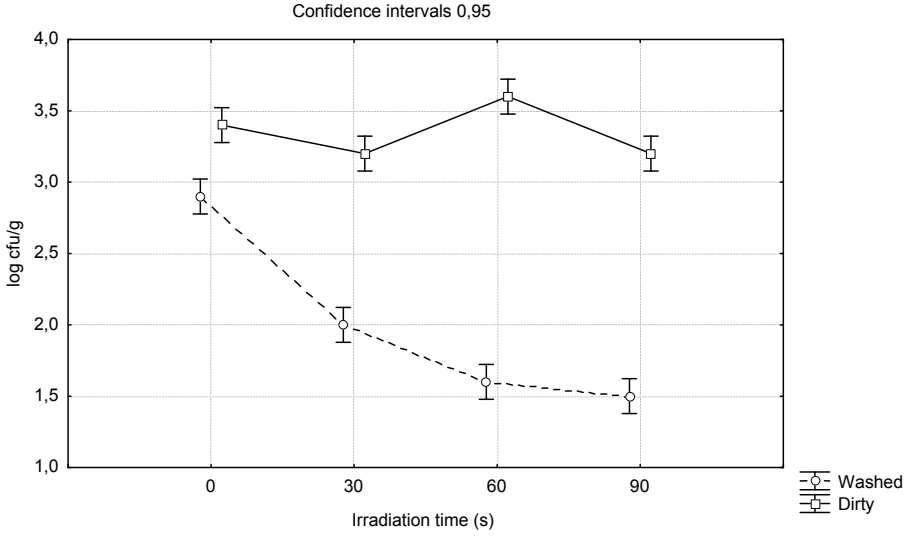

Fig. 2. The effect of UV-C irradiation time on counts of coliform bacteria per 1 gram shell

In the conducted studies no significant changes were found in the counts of coliform bacteria for eggs with soiled surface. This is probably connected with the low radiation hardness of UV-C radiation. Short wave UV radiation penetrates to the depth of 0.1 to 0.5 mm, while long wave penetrates slightly deeper to 2 mm [Bintsis et al., 2000]. Moreover, UV radiation is additionally absorbed by the organic material contaminating the shell surface, thus additionally reducing the effectiveness of sanitation by irradiation [Chaves et al., 2002].

In case of eggs with soiled surface after 60 s irradiation an increase in the count of coliform bacteria was observed, which was probably connected with the heterogeneity of the material or the specific character of the plate method. In this method all colonies cultured on the plate are counted. However, microorganisms are rarely found in the form of single cells, more frequently are retained after divisions in the form of diplococci, chains, etc. Treatment of bacterial cells with UV-C radiation at 254 nm results in the disruption of bacterial clusters into smaller ones. From each smaller cluster in the flooding method a single colony grows, thus probably the observed increase in the number of microorganisms after 60 s irradiation.

As a result of conducted analyses it may be concluded that sanitation of the surface of egg shells in case of eggs for human consumption using the combined washing and UV-C irradiation may be an effective and efficient method to eliminate pathogenic microflora from the shell surface. Results indicate the suitability of this method for the preparation of eggs for human consumption to retail sale.

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14

UV DESINFECTION AS A MEASURE TO ENSURE SUITABLE AIR HYGIENE IN FOOD PRODUCTION

Introduction

According to the food legislation "no dangerous food product can be introduced to the market" (178/02 art.14, item 1)[5]. And "the main responsibility for the safety of food lies in the enterprise of the food chain" (852/04 art.1, item 1a)[6]. It also signifies that "enterprises of the food chain are obliged to make sure that the foodstuffs were concordant with adequate microbiological criteria (…). For that reason on each stage of production, processing and distribution of food including retail trade, the enterprises of the food sector undertake measures within obligatory procedures based on HACCP rules and within the good practices implementation (…) in order to ensure: a) such a way of deliveries and processing of raw materials and foodstuffs, under the control of a given enterprise, as well as such conduct with them as to meet the criteria of the hygiene of the process; b) the possibility to meet food safety criteria to be applied within the whole period of validity of the products in possible to predict conditions of distribution, storing and use" (2073/05 art.3, item 1) [7]. Thus, to provide food safety one should start with providing safety to the raw materials and with conducting the production in adequate sanitary conditions. These conditions include hygienic habits of the personnel as well as adequate cleanliness of the production environment – including machines, devices, production areas and the air.

It is possible to obtain an adequate microbiological quality by the use of various methods of removing and eliminating impurities through hygienic activities, such as: cleaning, washing, disinfections and sterilization or by eliminating certain dangers from the production process.

Disinfection methods consist in chemical and physical disinfection. Chemical methods are based on the change of water activity, the accessibility of oxygen, the CO_2 level, the use of anti-microbiological substances, the change of redox potential. The advantage of using chemical disinfection is high effectiveness, whereas disadvantages include residues which can permeate to foodstuffs. For physical disinfection the temperature is used (usually high temperature), hydrostatic pressure, ultrasounds, magnetic field, ionizing radiation and light.

For air disinfection the mechanical methods are used consisting in applying various types of filters retaining pollution. Cotton fibrous filters are used, fiberglass and membranes. The air is filtered through the solutions of acids and lyes. Chemical methods of air disinfection are spraying pairs of gases and aerosols containing germicidal substances, such as e.g. peracetic acid, hydrogen peroxide, sodium hypochlorite, lactic acid, propylene glycol and its derivatives. For such a disinfection to be effective there must be an adequate air humidity provided. Sufficient sanitary care should be taken due to the fact that these

substances are toxic, allergenic or irritating for man. After the treatment there remain residues in the air, ventilating ducts and on surfaces, which then can have a negative influence on the product or employees but there is a method to this, which is very slow. [Kędzia, 1984] Physical methods of air sterilization are heating the air by compressing it to high pressures, electrostatic precipitation and the use of ultrasounds and various kinds of radiation. High-power cathode rays, gamma rays, ionizing radiation and UV radiation are used [Drewicz, 2000; Kunicki-Goldfinger, 2007].

For disinfection mainly ultraviolet light is used. It is an ultraviolet light, i.e. it has the length of the light wave below 400 nm. Visible light is in the band 400–730 nm. Ultraviolet radiation can be split into three bands. UVA with the longest waves (320–400nm) permeates glass and skin, causing the darkening of skin and "photoageing"; usually this length of waves is used in solariums. UVB has the 280–320 nm wave band, causes burns and skin injuries, but under the influence of this light vitamin D and melamine, i.e. skin pigment, form in the skin. In food industry this light is used in insecticidal lamps to attract insects. The shortest ultraviolet waves, UVC (210–280 nm), are considered to be deadly radiation. Natural UVC, originating from sun light, does not reach the surface of the Earth as it is stopped by an ozone layer [Kunicki-Goldfinger, 2007].

The UVC to be used by man is emitted by mercury burners constructed of a low-pressure quartz pipe filled with mercury vapors. Powerful antibacterial action exhibits radiation with 280–240 nm length and the optimum activity is 253.7 nm [Kędzia, 1984] Specific species and forms of microorganisms exhibit various UV radiation sensitivity. The most sensitive are vegetative forms of microorganisms. Viruses and spores of bacteria are from several to several dozen times more resistant to destroying them with the use of light. Very high resistance is exhibited by spores of mould, which are several thousand times less sensitive than vegetative forms of bacteria [Kędzia, 1984]. Bactericidal action upon a cell consists in inducing changes in DNA and RNA of a cell, generating a denaturation of proteins, enzyme-nucleotidase deactivation by toxic activity of the created free radicals and suboxides [Drewicz, 2000; Kędzia, 1984; Kunicki-Goldfinger, 2007]. The same toxic action is made by UV radiation on human cells and tissues, thus, it is essential to use shields protecting employees from harmful activity. The most powerful bactericidal activity is induced in oxygen conditions. The UVC disinfections can be applied to a surface, raw materials and air.

The effectiveness of air disinfection depends on the level of air pollution with mechanical particles (dust particles, dust), as the radiation stops on them and disperses without reaching the surfaces to be disinfected. Another determinant of effectiveness is an adequate choice of emitters to the volume of disinfected surfaces or air; it all concerns the fact that filaments give light to a limited surface and their effectiveness depends on their placement and disinfecting capabilities in a specific time interval. Another aspect which should be considered is the intensity of air movement what is connected with mixing or exchanging air masses. More factors are the time of radiation and the length of waves emitted by burners. It has been stated that during the time of use of the lamps the frequency of waves changes which results in decreasing the effectiveness of a disinfection. The last two factors affecting the disinfection efficiency are humidity of air and the temperature [Kędzia, 1984].

The UVC lamps are used to disinfect the air in health care centers. In this elaboration an attempt has been made to evaluate the effectiveness of the air in a meat production plant.

In every food industry plant the production areas can be divided into categories as far as the required microbiological purity of the air is concerned. One can distinguish: areas of low risk level – in places where the quality of the air does not affect the fastness or microbiological safety of a product, this concerns mainly such sections where raw materials or end products are insulated from the air, e.g. in storerooms or in extra-production zones. Areas of an average risk are the areas where the quality of the air constitutes an essential element contributing to an overall safety, and the air plays the main role in preventing secondary microbiological impurities. These zones are e.g. locks, areas used for the preparation of raw materials and pretreatment. The areas of a high risk are the areas where the quality of the air plays a critical role in preserving product safety. This concerns rooms where the goods are cooled with air or where they are packed, or where the air is used as a production raw material [Drewicz, 2000].

In a meat production plant there may be numerous sources of air pollution. They may be an inadequate sanitary condition of the plant, including machines and devices used for production, equipment, ventilating ducts and sewage system ducts. Another emitter of pollution, also air pollution, are the employees of a plant inducing masses of air while moving around and carrying impurities between the zones due to bad hygienic habits, the lack of personal hygiene and not observing the rules resulting from GHP and GMP. The very designing and constructing of the sewage system, ventilating and water network has a great influence on the transmission of impurities in a plant, including air pollution [Drewicz, 2000].

The microflora of the air in a meat production plant are cocci of the *Micrococcus* and *Sarcina* type, *Staphylococcus spp*, *Alcaligenes spp.*, *Bacilli spp*. In the air there may also occur spores of filamentous fungi of the *Cladosporium*, *Penicillium*, *Aspergillus*, *Alternaria*, *Botritis* or *Rhizopus* and *Mucor* types as well as yeast *Rodotorula*, *Torulopsis* and *Candidia*. The air may also contain pathogenic microflora [Drewicz, 2000].

Materials and methods

The tests were carried out in November 2008 – January 2009 in a meat production plant approved for a free trade on the territory of the whole European Union, i.e. meeting rigorous veterinary requirements, also in the sphere of hygiene. For the tests a room of the production of minced meat was chosen, where the hygiene of production, including the air, is especially essential due to the character of the product offered. The hall inspected was of the surface ca. 85 m^2 and capacity ca. 300 m^3.

The equipment of the hall included: weights, ice flake maker, mincers with mixing, as well as a forming-packing machine and an UV lamp. The ready made product was vacuum packed. The plant operates on a three-shift system of work, of which the production cycle of the inspected hall included two production shifts and one shift for cleaning the plant.

Considering the size of the hall and the way the production was carried out, for testing the efficiency of ultraviolet radiation an UV lamp was chosen, type E2000S made by an Austrian company SterilSystems, with built-over filaments and a forced air circulation. The air was sucked inside the device by an integrated axial ventilator, and then underwent

sterilization. The forced air circulation guaranteed the flow of 400 m^3/h, what was more that the cubature of the tested room. The casing of the lamp and the way of protecting the electronics in the lamp guaranteed its waterproofness and its resistance to humidity because to clean the hall the foam cleaning was used. For the whole time of the tests the lamp was switched off as it did not cause danger to employees.

The diagnostic method was the use of microbiological tests of the air at the use of the Koch sedimentation method for the overall number of microorganisms and the overall mould quantity. The results were treated in relation to the calculation of CFU in 1 m^3 of air.

In order to specify the influence and effectiveness of the UV radiation on the purity of air to ensure product safety, six measuring points were selected, located on different heights from the floor, in different places of the room. The characteristics of measuring points is given in Table 1, and the effectiveness connected with the operating cycle of the plant was presented in Table 2.

Table 1

The characteristics of measuring points

Measuring point	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Name of the place	Ice flake maker	Weight	Sink	Packing machine	Table	Entrance
Relative height	100 cm	0 cm	0 cm	70 cm	80 cm	0 cm

The activity in the measuring points was defined by using numerical definitions, where 0-no work, and the activity was described by giving the number of production processes taking place next to the measuring point, assuming that each activity affects the mixing of air masses. On the minced meat production hall the following were defined as production processes: ice production, weighing raw materials for production, mincing, stamping of packages, packing made by a packing machine, labeling and placing ready made packages of the product in containers. The act of opening of any door to the hall was also considered as an activity because it also caused the induction of air movements.

Table 2

The working cycle of the plant – the activity in measuring points (personal elaboration)

Hour/Measuring point	No. 1		No. 2		No. 3		No. 4		No. 5		No. 6	
	Before UV	After UV	Before UV	After UV	Before UV	After UV	Before UV	After UV	Before UV	After UV	Before UV	After UV
6.00.	0	0	1	0	0	0	0	0	1	0	1	1
9.00.	0	1	0	0	0	0	0	0	1	0	1	2
12.00.	2	1	1	1	1	1	2	2	0	1	2	1
15.00.	1	3	2	1	2	2	2	2	0	1	2	2
18.00.	2	3	2	2	2	2	2	2	0	0	1	1

From these points the smears were taken by using the sedimentation method. The fall time was 15 minutes. The inoculations were made onto already prepared Petri plates filled with ACH mediums for testing the number of moulds and PCA for testing the number of bacteria. The plates were delivered by an accredited laboratory, which also carried out the incubation and took readings of the results as well as performed necessary calculations and prepared reports from the tests.

The tests were carried out in two repetitions – before installing the UV lamp and seven weeks after installing it. One cycle of tests lasted 12 h during which every 3 hours from each point the inoculation was taken to examine the changes in the number of microorganisms during a production cycle. At carrying the tests the temperature of air in the production hall oscillated between 5,4–9,8°C, and the humidity was 45–84%. The highest humidity was in morning hours and with the course of time the humidity decreased.

The results obtained were analyzed statistically by using the Fisher LSD test at an assumed significance level $p \leq 0.05$. (Statgraphic software, ver. 5.1. for Windows).

Results and discussion

The overall number of microorganisms in the air was varied depending on the place (measuring point) and the hour of taking the measurements (Fig. 1). It resulted from the activity in specific points. In the second testing at 6:00 a.m. the number of microorganisms was greater than before installing the UV lamp – it was caused by cleaning the hall at 5:30 a.m. after the night shift what caused the microorganisms to be brought up to the air by water aerosols and later they started falling down. The general tendency was concordant with the one which was expected.

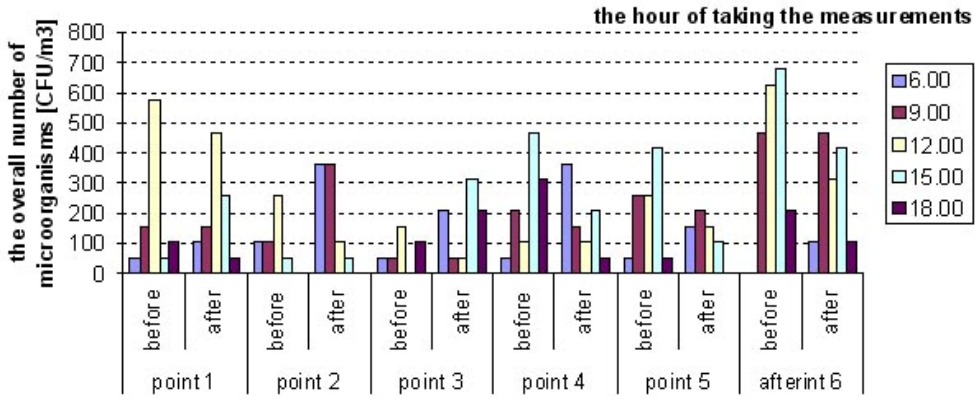

Fig. 1. The overall number of bacteria depending on the measuring point and the hour of taking the reading

The overall number of yeasts and moulds in the air was varied depending on the place (measuring point) and the hour of taking the measurements. The biggest pollution was recorded in the hours of the greatest activity of the plant – about noon, and in specific points depending on the use of devices and places, in which the measuring points were designated. The contamination with moulds and yeasts was much lower than with bacteria.

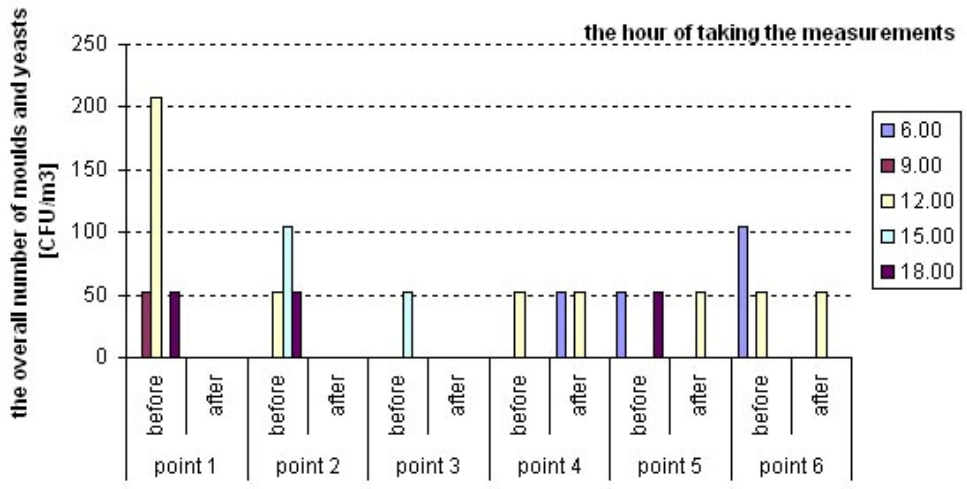

Fig. 2. The overall number of moulds depending on the measuring point and the hour of taking the reading

Figure 3 presents the influence of the use of the UV radiation on microorganisms – bacteria, moulds and yeasts.

The statistical analysis of the results obtained showed statistically significant influence of using the UV rays on the decrease of the number of microorganisms in the tested room - picture 3.

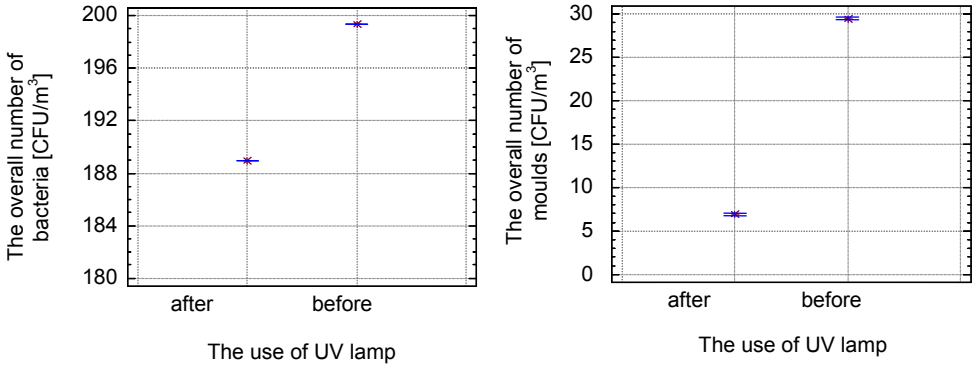

Fig. 3. The effectiveness of the UV radiation on air purity in an examined room

Conclusions

The literature available proved the influence of the UV radiation on the improvement of air hygiene – by the decrease of microorganism content – including bacteria, mould, fungi and viruses. However, the examinations carried out were made in room temperature. It was essential in the examinations to check the effectiveness in lower temperatures.

The test carried out indicated essential statistical decrease of air pollution in the production hall, and thus the effectiveness of the UV lamp for the disinfection of air in rooms with lowered temperature.

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15

ASSESSMENT OF MICROBIOLOGICAL QUALITY OF FOODSTUFFS INTENDED FOR PARTICULAR NUTRITIONAL USES

Introduction

Foodstuffs intended for particular nutritional uses includes, amongst others, infant and baby food up to the age of 3 [WE 2073/2005]. The manufacturers of the mentioned foodstuff are obliged to comply with particular conditions of production and introduction of their products to the market. They are also obliged to employ HACCP. Infant and baby food should be subjected to particular sanitary control measures on each stage if its production. Microbiological criteria relating to foodstuffs intended for particular nutritional uses, which are described in regulation by European Commission 1141/2007. The mentioned document determines allowed amount of microbiological contamination of food.

Microbiological safety criterium for foodstuffs intended for infants and babies consists of: *Listeria monocytogenes*, *Salmonella*, *Enterobacter sakazaki* and *Bacillus cereus*. According to above-mentioned document, the allowed level of contamination of foodstuffs intended for infants and babies with *Bacillus cereus* is $5*10^2$ cfu/g [WE 1441/2007].

The *Bacillus cereus* bacterias are a gram-positive sporous bacillus. They are able to produce heat-resistant spores and intensive decomposition of protein, which may generate toxic matter transformation products [Prośniak et al., 2006]. The mentioned bacterias are aerobic or aerotolerant. Most of these bacterias are mesophile of optimal temperature of development between 30 – 37°C and thermophile (over 50°C). There are also strains with psychrophile characteristics. The kind of *Bacillus* has vast composition of enzymes which enables them to use various sources of carbon and nitrogen. They develop in a broad pH range: 4-9 and their tolerance to NaCl concentration is 10%.

Bacillus cereus causes food poisoning after being consumed with food in an amount of $10^3 – 10^7$ of live cells or produced toxins, which irritate the bowel mucosa. These microorganisms are able to produce two types of toxines: heatsensitive – which causes diarrhoea, and thermostable which does not inactivate after being heated in the 120°C temperature for 30 minutes. This toxin is known as vomit toxin [Łaniewska-Trokenheim, 2007; Jay et al., 2005]. The symptoms of food poisoning caused by *Bacillus cereus* usually appear after from 30 minutes to 12 hours after being consumed with food containing mentioned toxins [Łaniewska-Trokenheim, 2007, Gładysz et al., 2006].

The origination of the *Bacillus cereus* is, among all, raw milk and its products, eggs, raw fruits, confectionery, desserts, puddings, creams, rice [Prosniak et al., 2006; Nicklin et al., 2000].

The presence of starch in a given food product favours pullulation of *Bacillus cereus* spores and production of toxines. These can cause a bitter aftertaste of food and ductility of bread [Lasik et al., 2001].

The origination of the food poisoning caused by *Bacillus cereus* include also meals which contain cooked or fried rice, beef, chicken, mussels and prawns [Gładysz et al., 2006, Novak et al., 2002].

Bacillus cereus also cause decaying of dairy products. The spores produced by the mentioned microorganism have strong hydrophobic surface and therefore are able to produce biofilms. Their significant immunity to detergents and disinfectants also favours the development and pullulation of spores. *Bacillus cereus* is difficult to complete elimination ingredient of raw milk, which in result can also be the cause of the presence of the bacteria in dairy products [Anusz, 2006; Berthold & Ramatowska, 2008].

The aim of the research is to asses the prevalence of *Bacillus cereus* in foodstuffs intended for particular nutritional uses; using as example ready meals for infants and babies.

Material and Methods

The research material included ready meals sold in jars; soups, main courses and desserts intended for infants and babies from 4 to 12 months old.

Five commercially available food products for children, supplied by five different manufacturers were tested (A, B, C, D i E). The research material included dishes which were suitable for immediate consumption (n = 42) and dishes which required further thermal treatment (n = 44). These included soups (n = 26), main courses (n = 18) and desserts (n = 42) intended for infants over 4 months old (n = 20), over 6 months old (n = 42) and over 9 months old (n = 24).

The size of the *Bacillus cereus* population was marked on manufactured by Merck Mossela base with added selective factor; egg yolk emulsion and polymyxin B antibiotic. The incubation of *Bacillus cereus* was carried out in the 32°C temperature during 18–40 hours period. The research samples were microbiologically analysed immediately after its arrival to laboratory. The research was carried out between May and July 2008.

Results and Discussion

The carried out research indicated contamination on levels between 0 and $9*10^3$ cfu/g, while 30% of the 86 samples indicated the presence of *Bacillus cereus*.

The highest percentage of samples which contained *Bacillus cereus* was amongst samples which contained cooke rice (22% of rice), ricestarch, cornstarch, vegetables (44%) and milk (50%). Morever, the reasearches carried out by Dolińska et al. and Feijoo et al. confirm the presence of *Bacillus cereus* in milk and dairy products [Dolińska & Berthold., 2008; Feijoo et al., 1997]. The mentioned samples included samples of soups, main courses and desserts and the amount of *Bacillus cereus* reached between $1*10^2$ and $9*10^3$ cfu/g.

Table 1

The amount of *Bacillus cereus* in soups, main courses and desserts intended for infants, expressed in cfu/g

The population of *Bacillus cereus* in infant food			
Sample	The population [cfu/g]	Average [cfu/g]	The percentage of researched material [%]
Soups	$0 – 3*10^2$	$83,6*10^0$	23,1
Main courses	$0 – 1,8*10^3$	$2,0*10^2$	44,4
Desserts	$0 – 9*10^3$	$4,4*10^3$	28,6

Among dishes which required further thermal treatment, the highly contaminated samples included main course meals containing around 44% of vegetables (carrot, green peas and tomatoes), cooked rice (22%) and chicken (8,5%). Significantly higher contamination with *Bacillus cereus* ($9*10^3$ cfu/g) occured in desserts containing cornstarch and fruits (46,5% apples, 37,5% peaches and 6% grape juice).

Analysis of soup samples indicated that around 23% of researched samples contained *Bacillus cereus* on levels between $1*10^1$ and $3*10^2$ cfu/g.

The results of microbiological research of the main course samples intended for infants indicated that over 44% of researched material contained *Bacillus cereus*. The contamination with this microorganism on a level of $1,8*10^3$ cfu/g was noted in 11% of the tested samples. The highest amount of the bacteria occured in samples which contained vegetables (38%) and cooked and minced rice. 22% of tested main course samples indicated contamination with *Bacillus cereus* on a level of $1*10^1$ cfu/g, and around 10% on a level of $2*10^1$ cfu/g. The ingredients of those main courses were similar (Fig. 1).

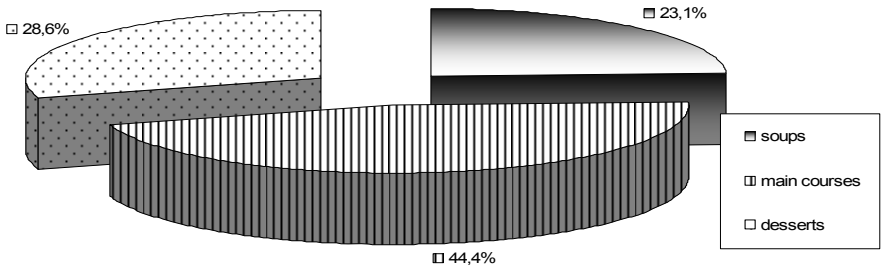

Fig. 1. Samples containing *Bacillus cereus* according to the type of sampled food

Microbiological analysis of dessert samples indicated contamination with *Bacillus cereus* between 0 and $9*10^3$ cfu/g. Almost 70% of tested samples did not contain *Bacillus cereus*. The contamination on a level of $1*10^2$ cfu/g was noted in 9,5% of tested desserts, while 14,3% contained *Bacillus cereus* on a level if$1*10^1$ cfu/g. These were the samples of desserts which contained fruit, cooked rice, ricestarch, wheat and oat flakes, and dried milk. Only 4,8% of samples were contained with *Bacillus cereus* on a level of $9*10^3$ cfu/g.

The highest level of contamination with *Bacillus cereus* was noted in samples produced by the manufacturers A and D, 50% of samples indicated the presence of the mentioned bacteria. Only the samples supplied by the manufacturer E were not contaminated with *Bacillus cereus*, which solely included dessert samples. Small amount of samples from manufacturer E was due to the limited availability of these products. The mentioned products were a novelty among baby food products available in the shops. The levels of contamination in products by manufacturer B and C were at 11% (B) and 18% (C) (Fig. 2).

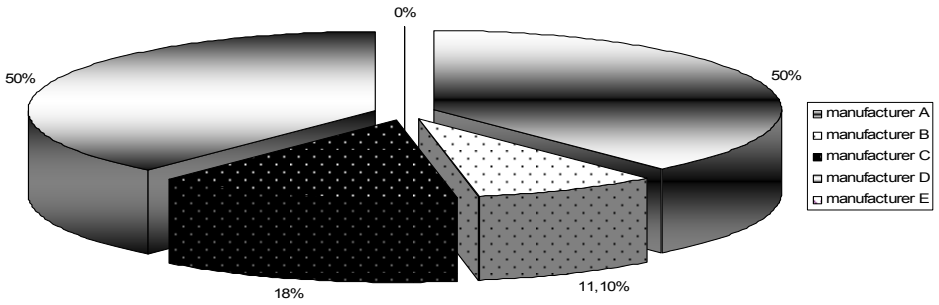

Fig. 2. Samples containing *Bacillus cereus* according to the manufacturer

The analysed material, which contained cooked rice or ricestarch was contaminated with *Bacillus cereus* on a level of 32,14%, while the samples which did not contain rice were contaminated with the mentioned microorganism on a level of 26,7%.

Infants and babies are a consumer group which is particularly prone to any types of infections and therefore food intended for that group should comply with high requirements of microbiological purity. The level of contamination with *Bacillus cereus* in the researched material was not hight, however in 4,7% of tested samples it exceeded the allowed limit ($5*10^2$ cfu/g). Considering the fact, that the amount which can couse food poisoning is between 10^3 and 10^7, the mentioned levels of contamination does note pose a danger. However, it is important to remember that those doses may cause symptoms of food poisoning among infants; especially dangerous diarrhoea or vomits. There is also a sparse data which suggests the influence of infection by *Bacillus cereus* toxines on the development of infants' nervous system [Lequin et al., 2005]. Therefore, it seems to be reasonable to carry out researches aimed at the determination of the risk of microbiological contamination of foodstuffs intended for children. It is also important to constantly monitor the sanitary conditions of every step of production of the mentioned food.

Conclusion

1. The highest level of contamination with *Bacillus cereus* was among samples which contained rice, vegetables and milk.

2. In the researched material, the very broad discrepancy of the size of the population of *Bacillus cereus* was caused by usage of samples of food produced by 5 different manufacturers

3. 4,7% of the researched material contained *Bacillus cereus* on a level exceeding allowed limit.

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Ustawa z dnia 25 sierpnia 2006 r. o bezpieczeństwie żywności i żywienia, Dz. U. z dnia 27 września 2006.–2. Rozporządzenie Komisji (WE) nr 1441/2007 z dnia 05 grudnia 2007 r. zmieniające rozporządzenie (WE) nr 2073/2005 w sprawie kryteriów mikrobiologicznych dotyczących środków spożywczych.

16

COMPARISON OF MICROBIOLOGICAL AND BIOCHEMICAL PROFILES OF POLISH BLUE-VEINED CHEESES MANUFACTURED IN DIFFERENT DAIRIES

Introduction

Blue-veined cheeses differ from other kinds of cheese with their exceptional appearance and unique taste and flavor. The mould *Penicillium roqueforti* overgrowing a cheese core determines their characteristic appearance. During ripening process extensive proteolysis and lipolysis takes place due to the high activity of hydrolytic enzymes secreted by *P. roqueforti* moulds [Grippon, 1993]. The products formed during protein and fat decomposition are mainly responsible for the original organoleptic properties of that kind of cheese.

The most famous blue-veined cheese is French Roquefort, which is usually prepared from sheep milk alone or sheep milk with little addition of cow milk [McSweeney P., Sousa M., 2000]. Other popular European blue-veined cheeses are: Italian Gorgonzola, English Stilton, Danish Danablu and Spanish Cabrales. In Poland the most popular blue-veined cheese is Rokpol, a Roquefort-type cheese, produced from cow milk. Only few Polish diaries produce Rokpol cheese. Since high quality of cheeses is getting more and more important to the consumers we investigated the ripeness and quality of Polish blue-veined cheeses originating from three different manufacturers.

Materials and Methods

Rokpol cheese produced by three different Polish dairies (A, B, C) were analyzed. For experimental purposes ripened Rokpol cheese were purchased (eight samples from each manufacturer) in the same time.

Microbiological analysis

For microbiological analysis samples (10 g) from the core and surface layer (0,5 cm deep) were homogenized for 2 min in a Stomacher 400 Lab Blender (Seward Medical, London, England) in 90 mL of 2% sodium citrate. Consecutive decimal dilutions were prepared and plated in duplicate on specific media for total viable counts of: mesophilic bacteria (Plate Count Agar, Difco), mesophilic lactobacilli (MRS Agar, Oxoid) and lactococci (M17 Agar, Difco). The media for bacteria counting contained cycloheximide at a concentration of 100 μg mL^{-1} to inhibit yeast growth. Moulds and yeast were determined on Oxytetracycline-Glucose-Yeast Extract Agar (OGY, Oxoid) composed of 2.0% glucose, 0.5% yeast extract, 0.01% oxytetracycline-HCl, and 1.5% agar. For yeast counts the medium was additionally enriched with 0.2% sodium propionate to suppress mould growth. Plates were incubated for 2–5 days at 30°C. Results are presented in log values.

Chemical analyses

Total solids (TS), fat and protein contents in cheeses and their pH were estimated according to Zmarlicki [1981]. Protein degradation in cheeses was measured by determination of water soluble nitrogen (WSN) and total nitrogen (TN) by Kjeldahl method. Also, contents of free amino groups in fraction soluble in water [Kuchroo and Ramilly, 1982] and phosphotungstic acid [PTA, Jarret, 1982] were determined according to Kuchroo and Ramilly [1983]. Electrophoretic separation of proteins was performed according to Andrews [1983].

Biogenic amines extracted from the cheeses according to method of Etter et al. [1990] modified by Bütikofer et al. [1990] were dansylated with dansyl chloride (50 mg/mL in acetone). The separation of dansylated amines (5 uL) was performed at 37°C using a column MERCK LiChroCart HPLC 3 Purospher RP-C18, 5 μm, 150 mm. The solvents were as follows: buffer pH 8.0: Tris 0.1M pH 8.0 /acetic acid 0.1 M / water (2/1/2); solvent A: buffer pH 8.0 (30 mL) / acetonitrile (550 mL) / water (420 mL); solvent B: buffer pH 8.0 (2 mL) / acetonitrile (900 mL) / water (100 mL). Dansylated amines were detected at 254 nm. Identification of the peaks was done by comparison of their retention time with standard amines mixtures. The biogenic amines were quantified by referring to calibration curves calculated from the analysis of a commercial amines mixture with increasing known concentrations (8 μg to 1 mg/g of cheese equivalent) in 0.02 M sulfuric acid .

Free fatty acids (FFA) were extracted from cheese using the method of Deeth et al. [1983]. Acetyl chloride was used as methylating reagent. The separation was performed using a gas chromatograph (Agilent Technologies) equipped with mass detector (GC/MS), capillary column (Agilent DB-224 MS) parameters 60 m × 250 μm × 0,25 μm. The injector temperature was raised from 70°C to 240°C at a rate of 4°C min^{-1}. The flow rate of gaseous carrier (helium) was 2.0 mL/min and the split flow ratio was 1:100 for all investigations. The peaks were identified and quantified by reference to FFA standards. The relative fatty acids composition was estimated as a percentage of the total peak area.

Statistical analysis

The data were analysed statistically using Statistica AGXP V.5.5 The mean values with standard deviations are reported.

Results and Discussion

Table 1 shows the principal microbial groups determined in Rokpol cheeses manufactured in three Polish dairies (A, B, C). The number of moulds, yeast, total mesophilic bacteria and lactic acid bacteria (*lactococci* and *lactobacilli*) was analysed on the surface and in interior of the cheeses. Generally, the number of all analysed microbial groups was higher in the cheeses' core than on their surface, with the exception of yeast, which in all samples grew better on the surface. In cheeses from dairy C number of yeasts on the surface was the highest and reached the level of 8.84 log cfu/g, while in cheeses from dairy A and B was lower by 0,5 and 1,5 log cfu/g, respectively. In analyzed cheeses in the core layer population of this group of microorganisms was similar and ranged from 5,52 to 6,08 cfu/g. Gobetti et al. [1998] found that yeast population in Italian Gorgonzola was constant at about 7,50 log cfu/g in both, surface and interior layers, while Gonzales et al. [1992] detected yeasts in interior of Spanish Gamonedo on the level of 7,32 log cfu/g . The number

of moulds in the core of Rokpol cheeses from dairy A, B and C was: 7,74; 8,35; and 7.58 log cfu/g, respectively. On cheese surface population of moulds was lower by ca. 2,0 logarithmic cycles in products originating from first two dairies and by 0,5 logarithmic cycle in cheeses from third dairy C. The results observed for Rokpol cheese did not differ significantly from those described in other blue–veined cheeses [Gonzales et al.,1992; Gobetti et al.,1998]. Total mesophilic bacteria as well as lactic acid bacteria determined both in M17 and MRS were present in all cheeses at high levels. The populations of these microbial groups differed by 0,5–2,5 as compared cheeses from three tested dairies.

Table 1

Total counts of the principal microbial groups occurring in Polish Rokpol cheeses originating from three dairies (log cfu/g)

Part of cheese	Dairy	Moulds	Yeasts	*Mesophilic* bacteria	*Lactoccocci*	*Lactobacilli*
Surface	A	5,94±0,25	7,19±0,20	6,32±0,26	6,10±0,25	7,76±0,11
	B	6,41±0,26	8,33±0,37	7,64±0,15	7,87±0,35	6,49±0,24
	C	7,07±0,15	8,84±0,13	9,52±0,63	9,16±0,23	7,56±0,16
Interior	A	7,74±0,34	5,53±0,14	8,39±0,51	8,18±0,23	7,79±0,21
	B	8,35±0,27	6,08±0,54	8,15±0,26	8,45±0,12	8,45±0,30
	C	7,58±0,64	5,52±0,35	7,04±0,31	7,52±0,37	7,81±0,42

±SD (standard deviation)

Table 2

Mean values of gross composition (g/100g) and pH of Rokpol cheese originating from three dairies

Dairy	Total solids [%]	Protein [%]	Fat [%]	NaCl [%]	pH
A	57,58±0,70	20,70±0,64	31,5±0,80	2,91±0,14	5,72±0,21
B	55,18±0,33	19,98±0,36	27,8±0,90	5,40±0,11	6,09±0,08
C	54,62±0,92	21,19±0,53	28,9±0,90	2,71±0,08	5,77±0,34

±SD (standard deviation)

Table 3

Biogenic amines content in Rokpol cheeses originating from three dairies (mg/kg)

Biogenic amines	Dairy		
	A	B	C
Tryptamine	63,86 ±0,36	215,10 ±0,59	91,94 ±0,54
β-phenylethylamine	24,44 ±0,73	68,33 ±0,96	25,03 ±0,63
Putrescine	5,78 ±0,63	27,73 ±0,77	7,49 ±0,85
Cadaweryne	3,88 ±0,44	17,11 ±0,48	4,56 ±0,89
Histamine	275,47 ±0,47	237,14 ±0,63	197,02 ±0,93
Tyramine	6,00 ±0,78	33,98 ±0,47	33,98 ±0,94
Spermine	6,21 ±0,36	47,38 ±0,85	47,38 ±0,72
Spermidyne	9,16 ±0,86	9,16 ±0,49	53,21 ±0,68
Total	394,80	655,93	460,61

±SD (standard deviation)

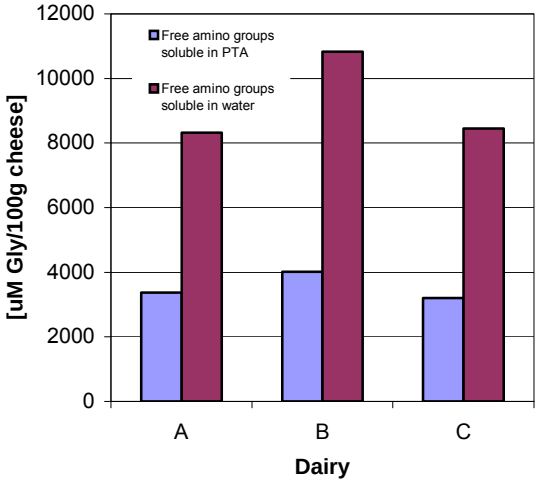
The principal chemical composition and pH values of analysed Rokpol cheeses are shown in Table 2. The mean value of total solids in cheeses was from 54.62 to 57.58% and differed by 2–3% with the manufacturer. These contents appeared lower than those obtained for other blue-veined cheese varieties such as Cabrales [Alonso et al.,1987], Stilton [Muir et al., 1995], but similar to those found in Danish Blue and Gorgonzola [Muir et al., 1995].The levels of protein in all cheeses were 19.98 to 21.20, while the fat content ranged from 27.28 to 31.50%. The NaCl values (2,63–2,91%) were similar for all analyzed cheeses , but lower than those observed in other blue-veined cheeses [Prieto et al. 2000] . The pH of cheeses from dairy A and C were similar (ca. pH 5,70), but lower comparing to cheeses from dairy B (pH 6,09). These values were also lower than those reported for other blue–veined cheeses such as Stilton, Cashel and Gorgonzola, but similar to Danablu [Zarmpoutis et al., 1997; Gobbetti et al., 1998].

The extent of proteolysis in Rokpol cheeses produced in different dairies was investigated quantitatively by determination of water soluble nitrogen and free amino groups in fraction soluble in water and phosphotungstic acid (Fig. 1). The lowest levels of protein degradation products were observed in cheeses from dairy A and the highest in cheeses from dairy B. WSN contents in analyzed cheeses were in range from 33,95 to 44,11% of total nitrogen. These values appeared similar to those observed in Irish blue veined cheeses, but considerably lower than in Stilton, Gorgonzola and Danablu [Zarmpoutis et al., 1997]. The free amino groups contents in Rokpol cheeses reached high level of 8 000–10 000 μM Gly/100g in fractions soluble in water. Their contents in fractions soluble in phosphotungstic acid, which are measure of very short peptides and free amino acids, were 29,11–37,03% of those determined in fractions soluble in water.

Protein degradation in Rokpol cheeses was also monitored electrophoretically (Fig. 2). Urea-PAGE of the whole cheese and of fraction unsoluble in water showed comparable level of casein degradation in all analyzed cheeses. Deeper proteolytic changes were observed in α_{s1}–casein fraction, especially in cheeses from dairy C. Significant differences between cheeses were also discovered in the pattern of soluble in water fractions. The highest number of bands resulted from casein degradation was shown also in cheeses from dairy C. These results confirm that great extent of proteolysis underwent in Rokpol cheeses similar to other blue-veined cheese varieties, which is caused by proteases originating from different sources: milk, primary starters and secondary starter es. *P. roqueforti*. Moulds are major source of hydrolytic enzymes in blue cheeses [Gobetti et al., 1998].

The content of biogenic amines was also determined in Rokpol cheeses. The presence of biogenic amines in cheese is attributed to the decarboxylating activity of microorganisms. Schneller et al. [1997] confirmed that starter cultures had great impact on biogenic amines formation in cheese. However their formation is affected by different factors, such as: raw milk quality, cheese microflora composition, synergism between different species, proteolysis, salt content, the pH and ripening temperature [Valsamaki et al., 2000]. All main biogenic amines were detected in analysed cheeses, however their concentrations differed depending on cheese origin (Table 4). In all of them quantitavely the most important biogenic amines were: histamine (197,02–275,47 mg/kg) and tryptamine (63,86–215,10 mg/kg). Generally, the highest concentrations of biogenic amines were observed in cheeses from dairy B (655,93 mg/kg), in which free amino content was also higher than in cheeses produced by two other manufacturers.

a/



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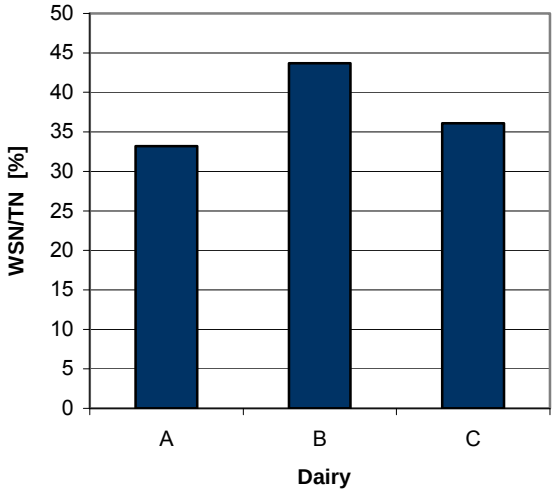

Fig. 1. Concentrations of free amine groups (a) and WSN (b) in Rokpol cheeses originating from three dairies

Scale of lipolysis in analyzed cheeses was monitored by determination of free fatty acids. As shown in Table 5 the highest release of these acids occurred in cheeses from dairy B, in which their contents was 5675,70 mg/kg. In cheeses from each dairy oleic acid reached the highest concentration, followed by miristic, palmitic, and stearic acids. Generally hydrolysis of triacylglicerols in blue-veined cheeses is more advanced comparing to other kinds of cheese [McSweeney & Sousa, 2000]. It is resulted from high activity of *P. roqueforti* which secrets two lipases acid and alkaline [Gripon, 1993]. However, also non starter microflora, especially yeasts, which are present in blue cheeses at high level, may affect lipid degradation process [Wojtatowicz et al., 2001].

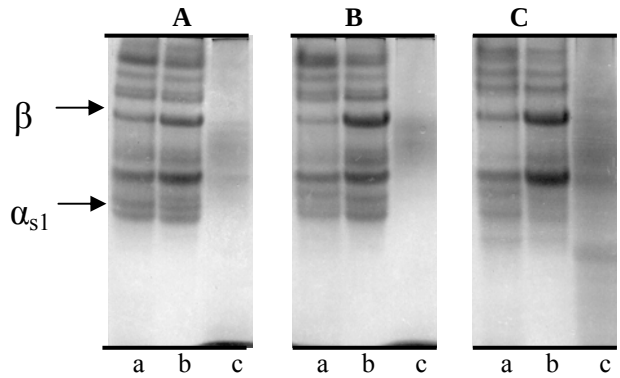

Fig. 2. Urea-polyacrylamide gel electrophoresis of whole cheese (a), fraction unsoluble in water (b) and soluble in water (c) of Rokpol cheese originating from three dairies

Conclusion

Rokpol is typical blue-veined cheese, in which process of protein and lipids degradation is advanced. Differences between cheeses manufactured in three dairies at the levels of this degradation processes may be resulted from differences in the ripening conditions as well as the enzymatic activity of starter and nonstarter microflora.

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17

STUDIES ON THE EFFECT OF MEATINESS ON INTRAMUSCULAR FATNESS IN SELECTED HAM, SHOULDER AND LOIN MUSCLES

Introduction

Long-term efforts of breeders and slaughterhause procurement services, using research and scientific advances in genetics and feeding as well as meat technology, have lead to a considerable increase in meat content in the carcass and reduced fatness [Borzuta et al. 2003; Blicharski et al. 2004; Migdał et al. 2004]. A lower carcass fatness is not only connected with thinner backfat, but also lower contents of inter- and intramuscular fat, referred to in international nomenclature as IMF [Różycki 2005; Blicharski et al. 2006]. IMF is deposited at the slowest rate in animal organisms. Its optimal level in loin is considered to be 2 to 2.5% [Eikelenboom et al.1996]. It constitutes an important attribute of meat quality and is found to be highly desirable both on the part of processing industry and consumers. Intramuscular fat has a positive effect on tenderness, flavour and juiciness and it reduces losses during thermal processing [Kirchheim et al.1997; Blicharski et al. 2004; Fortin et al. 2005]. Kirchheim et al. [1997] found significant correlations between tenderness and fat content in meat amounting to 2.5% ($r = 0.45^{**}$).

The observed interdependence between the level of intramuscular fat and the organoleptic value of meat was the reason why researchers started to search for its genetic background. It was attempted to identify and locate in the porcine genome QTLs determining intramuscular fat content. De Koning et al. [1999] showed [cited after Blicharski et al. 2004], that regions of chromosome 7 as well as 2, 4 and 6 may contain genes affecting the level of intramuscular fat. In literature we may find several methods facilitating estimation of meat marbling in pigs, e.g. computer analysis of LD muscle sections [Faucitano et al. 2005], ultrasound spectral analysis [Mörlein et al. 2005] or measurements taken using a spectroscope [Altmann and Pliquett 2006]. IMF is easy to recognize at muscle sections, particularly in loin, since it forms the so-called meat marbling. Some studies showed a high interdependence ($r = 0.51^{**}$ and $r = 0.61^{**}$) between IMF content and marbling in LD muscle [Rybarczyk et al. 2005; Faucitano et al. 2005]. In turn, reports on dependencies between marbling of different muscles and carcass meatiness are scarce.

The aim of the study was to assess marbling in six selected muscles depending on the level of meat content in carcasses of commercial fatteners.

Material and methods

Studies were conducted on 129 pig carcasses from the market population of the Bydgoskie, Poznańskie and Lubelskie regions, composed of equal proportions of gilts and hogs. At 24 h after slaughter left half-carcasses were dissected into primal cuts following the methodology of Walstra and Merkus [1996] and dissection of these cuts was performed, in the course of which m. *longissimus dorsi* (LD) was cut from loin, m. *biceps femoris* (BF), *m. semimembranosus* (SEM) and *quadriceps femoris* (QF) were cut from ham and m. *triceps brachii (TB)* was cut from shoulder. Marbling was assessed on cross-sections of these muscles and at the cross-section of m. *gluteus medius* (GM). The degree of fatness in these muscles was determined according to Canadian and American standards [Wise 1981; Kauffman et al. 1992] in a 5-point scale (1 point – slight fatness, 5 points – very strong fatness). Marbling in 6 selected muscles was evaluated in the following experimental groups, differing in meat content in the carcass, as established by dissection: group I over 55% (n = 45 head), group II 45–55% (n = 59 head), group III below 45% (n = 25 head).

Results were analyzed statistically, to obtain means, standard deviations and a two-way analysis of variance. Significance of differences between groups was calculated using the Tukey test. Moreover, linear correlation coefficients were calculated between marbling of analyzed muscles [Stanisz 1998].

Results and discussion

Mean results of marbling determination listed in table 1 indicate a rather high variation depending on the level of carcass meatiness and the type of tested muscles. This was confirmed by the analysis of variance, which results are given in Table 2. Statistical computations showed that both analyzed experimental factors have a highly significant effect on the level of marbling. The highest marbling, equivalent to approx. 3.2 points, was observed in muscles of fat fatteners from group III, classified to grades O and P, while it was lower in fatteners with high meatiness of grades E and S (Fig. 1).

Table 1

Mean results of muscle marbling scores depending on meatiness of pig carcasses (points)

Name muscle	I group over 55%		II group 45–55%		III group under 45%		Total muscle	
	$\bar{x}$	S	$\bar{x}$	S	$\bar{x}$	S	$\bar{x}$	S
m. Longissimus dorsi	1,77	0,69	2,35	0,76	3,30	0,97	2,34[a]	0,95
m. Biceps femoris	2,36	0,51	2,78	0,68	3,38	0,79	2,76[b]	0,74
m. Semimembranosus	2,06	0,61	2,42	0,69	3,04	0,75	2,42[a]	0,75
m. Quadriceps femoris	1,60	0,43	1,84	0,51	2,34	0,72	1,86[c]	0,59
m. Triceps brachii	2,01	0,63	2,31	0,76	3,14	1,04	2,38[a]	0,88
m. Gluteus medius	1,93	0,52	2,38	1,20	3,70	1,14	2,48[a]	1,18
Total groups	1,95[A]	0,61	2,35[B]	0,84	3,15[C]	0,99	2,37	0,91

A,B,C – significance differences between meatiness groups at P≤0,01
a,b,c – significance differences between muscles at P≤0,01

Table 2

Results of analysis of variance for marbling scores in the muscle x meatiness groups system

Type of variation:	Number of degrees of freedom	Sum square of deviations	Mean square of deviations	Value F	P
Overall	1	4142,93	4142,93	7260,54	0,000
Meatiness	2	135,58	67,79	118,80	0,000
Muscle	5	52,95	10,59	18,56	0,000
Muscles x meatiness	10	12,80	1,28	2,24	0,014
Experimental error	742	423,39	0,57	–	–

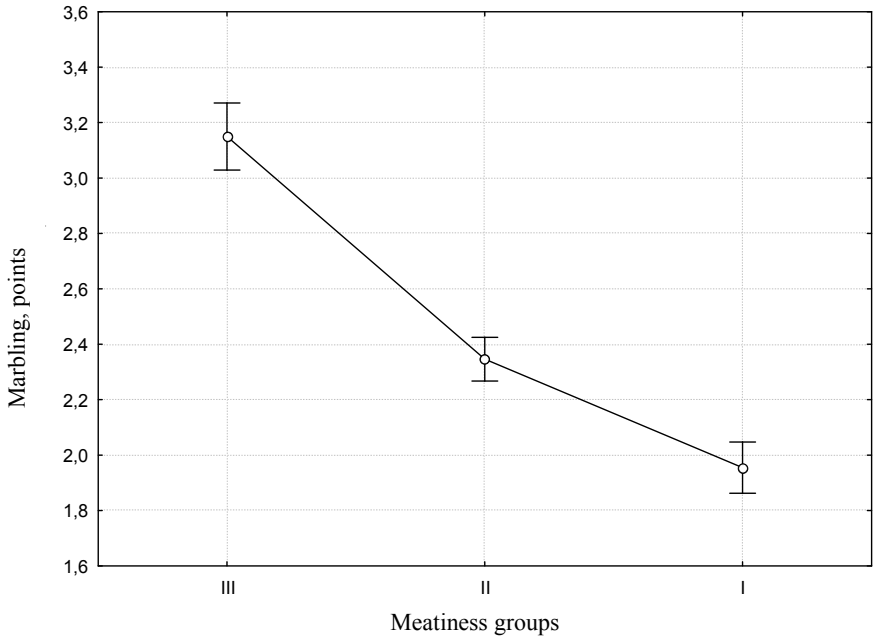

Fig. 1. Graphic characteristics of muscle marbling depending on the level of meatiness in pig carcasses

Recorded results are confirmed by literature data. Wajda [2008] stated that the proportion of intramuscular fat, which is closely correlated with marbling score.In the LD muscle it was two times higher in fatteners of grade O than those of grade E (2.50 and 1.32%, respectively). Similarly, the highest proportion of fat in carcass grades with low meatiness was observed by Wajda et al. [1995 and 2005], as well as Kortz et al. [2003] and Daszkiewicz et al. [2005].

Results confirmed also that marbling varies in individual meatiness grades. The lowest degree of intramuscular fat was found in the *quadriceps femoris* muscle (1.86 points), while the highest in the *biceps femoris* muscle (2.76 points). In the other analyzed muscles (*semimembraneous, longissimus dorsi, triceps brachii, gluteus medius*) marbling levels were intermediate between the above mentioned muscles (approx. 2.4 points). The level of mus-

cle fatness is comprehensively presented in Fig. 2, where we can clearly see the three-level system of marbling in six analyzed muscles.

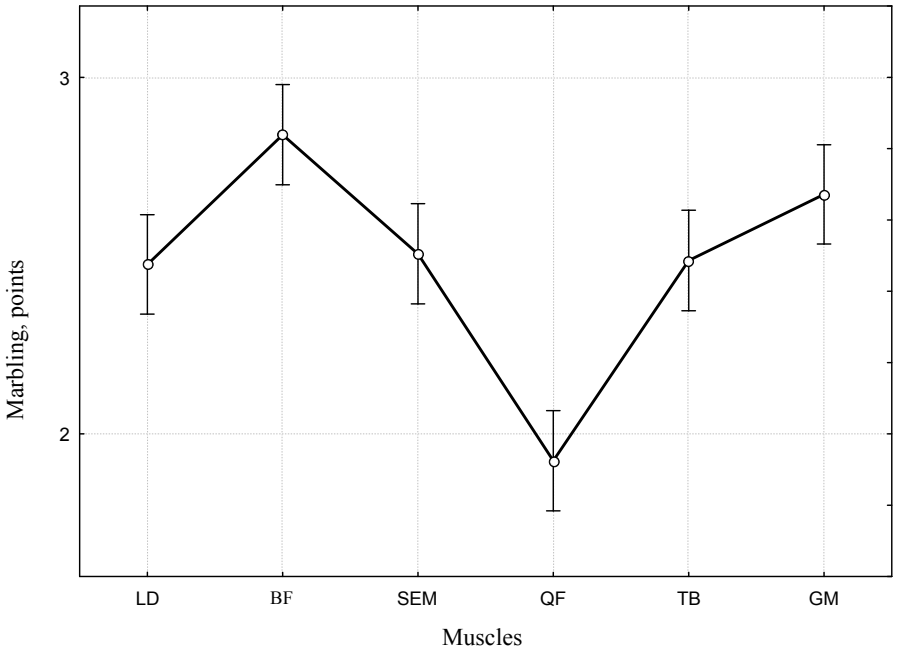

Fig. 2. Graphic characteristics of differences in marbling of analyzed muscles

Differences in marbling between muscles are relatively not well documented in literature. However the opinion that e.g. the *quadriceps femoris* muscle is characterized by the lowest fat content is well know. Intramuscular fat differs markedly from fat found inside muscle fibres and fat located in connective tissue membranes. As it was reported by Suzuki et al. [2005], a relationship between intramuscular fat and connective tissue membranes is indicated by a significant correlation (r = 0.43) between IMF and collagen content [cited after Blicharski et al. 2006]. It may be assumed that these dependencies are responsible for the variation in marbling of muscles.

Collected results indicate that the direction of changes of the marbling level, depending on the meatiness of the carcass, is different in all analyzed muscles, as it is confirmed by a significant meatiness group x muscle interaction ($P \leq 0,05$). It is seen in Fig. 3, where in each meatiness group the system of differences between muscles is similar but the differences are bigger between group III and the other groups than between I and II groups.

The paper also presents interdependencies between marbling of evaluated muscles (Tab. 3). Higher correlations were recorded between marbling in m. SEM and the BF, QF and LD muscles (r = 0.68 to 0.75), while they were lower between m. GM and the other muscles. This means that the GM muscle used in the analyses is a worse indicator of intramuscular fatness than e.g. such muscles as LD, SEM and BF.

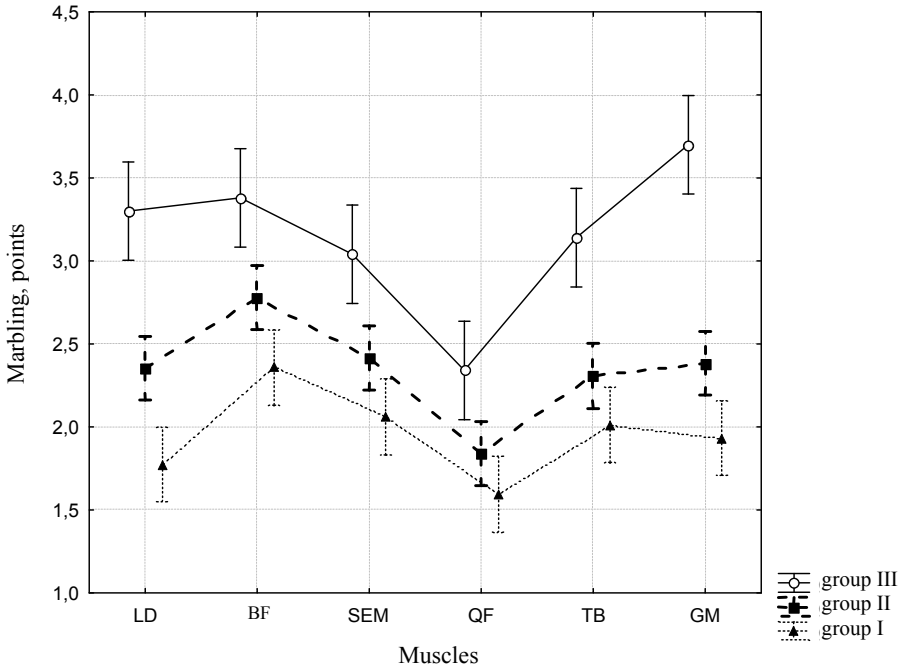

Fig. 3. Graphic characteristics of marbling depending on the level of meatiness of the carcass and the type of analyzed muscles

Table 3

Correlation coefficients between marbling of different pig muscles

Muscle	LD	BF	SEM	QF	TB	GM
LD	–	0,6168**	0,6772**	0,5445**	0,5987**	0,5860**
BF	0,6168**	–	0,7508**	0,5798**	0,6439**	0,5294**
SEM	0,6772**	0,7508**	–	0,6807**	0,6386**	0,5664**
QF	0,5445**	0,5798**	0,6807**	–	0,6578**	0,4548**
TB	0,5987**	0,6439**	0,6386**	0,6578**	–	0,5127**
GM	0,5860**	0,566**	0,5664**	0,4548**	0,5127**	–

** $P \leq 0,01$

Summing up the obtained results we may state that the level of marbling has a significant effect both on meat content in the carcass and the type of analyzed muscle. An increase in marbling was observed in muscles with a reduction of carcass meatiness. Among analyzed muscles the lowest marbling was recorded in m. *quadriceps femoris* and the highest in m. *biceps femoris*. Obtained results may be helpful in the selection of raw material for the production of high quality products as well as modifications of purchasing policy concerning slaughter animals consistent with the needs of meat processing plants.

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18

RISK ASSESSMENT OF *LISTERIA MONOCYTOGENES* GROWTH IN TVAROG CHEESE

Introduction

Predictive microbiology is a new trend in food microbiology, which is useful in predicting growth, survival, or inactivation of microorganisms in food products [Whiting & Cygnarowicz-Provost, 1992; Kołożyn-Krajewska & Jałosińska-Pieńkowska 1999; Kołożyn-Krajewska, 2000, 2003; Kajak, 2001].

The basic assumption of predictive microbiology is that growth of microorganisms is a function of food as an environment. The reaction of bacterial population to environmental factors is reproducible and these factors allow to determine the potential growth or inactivation of microorganisms from tests carried out in the past [Kołożyn-Krajewska & Jałosińska-Pieńkowska, 1999, 2003; Kołożyn-Krajewska, 2000; Kajak, 2001; McMeekin et al., 2002; Kowalik et al., 2004; Pinon et al., 2004].

Predictive models are concerned with determining microbiological growth factors such as temperature, pH, water activity (a_w), sodium nitrate and organic acids content, or atmospheric composition (aerobic, modified, vacuum) [Wijtzes et al., 1998; Baranyi, 2002; Kołożyn-Krajewska, 2003; Kołożyn-Krajewska & Jałosińska-Pieńkowska, 2003; Kowalik & Ziajka, 2005].

Predictive models may be applied to designing new products, cause they allow to estimate potential hazards, selection of the most appropriate preservation method, and determination of storage possibilities and conditions. They can also be useful to estimate the consequences of possible incompatibilities in food production process and storage, to develop the bases for guides, standards, and criteria, or to determine the critical parameters in the critical control points of the Hazard Analysis and Critical Control Points (HACCP) system and play an important role as an educational tools for employees of industry and control quality [Whiting & Cygnarowicz-Provost, 1992; McMeekin & Ross, 2002; McMeekin et al., 2002; Kołożyn-Krajewska & Jałosińska-Pieńkowska, 2003].

Therefore, the application of computer software for modeling of microorganisms growth in various food products and creation of predictive models play an important role and is an inseparable part of food microbiology. There is a lot of software and package software useful to predict behaviour of different groups of microorganisms in conditions that simulate food products.

Institute of Food Research (IFR) in the UK created add-in to Excel (MS Office) called Dynamic Modeling Fit (DMFit; (http://www.ifr.bbsrc.ac.uk /safety/DMFit/default.html).

Growth Predictor (GP) software was created by Food Standards Agency (FSA), IFR, USDA and Australian Food Safety Centre of Excellence. The aim of this program is to collect data to predict the reaction of microorganisms as a response to environmental food

conditions. Models in GP content data from studies conducted on many pathogens in variety of environmental condition (modified medium with different salt, organic acid, CO_2 content and pH and a_w). GP consist of many microorganisms' growth curves, which were obtained from studies and publications [Baranyi and Roberts, 1994; http://www.ifr.ac.uk/safety.growthpredictor].

DMFit and Growth Predictor were constructed according to Baranyi model [Baranyi and Roberts, 1994; Baranyi, 2002]. This is a dynamic model which describes the growth of microorganisms.

The period of lag phase is defined as a time necessary for synthesis of the substrate **q**, which is essential to microorganisms growth. This is so-called „work to be done". After this adaptation time, logarithmic growth phase starts and bacterial cells intensively multiply in this time. The microorganisms growth ends with the death phase, where the population decreases linearly.

$$dx/dt=[(q(t)/q(t)+1)\mu_{max}][1-(x(t)/x_{max})^m]\ x(t)$$

where:
x – the number of cells at time (t),
x_{max} – the maximum cell density,
m – the parameter characterizes the curvature before the stationary phase,
q(t) – the concentration of limiting substrate, which changes with time (t) :

$$dq/dt=\mu_{max}\ q(t)$$

[McKellar & Lu, 2004].

Much information about the growth of microorganisms in specific food product is available from database called Combase (www.combase.cc).

In recent years, outbreaks of infections caused by *L. monocytogenes* have been observed in an industrial society [Gliński et al., 2003; Kowalik & Ziajka, 2005]. Therefore, interest of this pathogen has increased not only for public health, but for economic reasons as well. *L. monocytogenes* is an environmental pathogen which can occur in milk and dairy products, and is of special concern because it can grow at refrigeration temperatures, and can cause food poisoning. Although other ways of transmission exist, food products have been clearly identified as a primary source of infection. Therefore, it is very important to control food products on the presence of *Listeria monocytogenes* in order to avoid outbreaks of food poisoning [Membre et al. 1997, 1999; Trafiałek et al., 2004].

Changes in consumer preferences have increased an interest in the presence of *Listeria monocytogenes* in dairy products. Recently, in the European Union countries the consumption of less processed, ready-to-eat, and fresh-like food has increased and more infections caused by *L. monocytogenes* have been observed. For example, in the European Union in 2006 the number of listeriosis was 1583, but in the USA at least 1600 cases every year is noticed (www.EFSA.europa.eu).

Listeriosis occurs infrequently with an incidence rate of less than 10 cases per million; however the fatality rate is high (general 20–40%), up to 75% in highly susceptible individuals, such as those with an immuno-compromised system [Le Marc et al. 2002; Norrung et al., 1999].

Virulent strains of *L. monocytogenes* may penetrate the gastrointestinal epithelium and enter phagocytic host cells where the bacteria is able to survive and multiply, thereby permitting access to the brain or the fetus in pregnant women, and may lead to death [Norrung, 2000; Roche et al., 2003; Gliński et al., 2003; Osek, 2005].

The presence of *L. monocytogenes* in raw milk and dairy products is a consequence of the widespread occurrence of this microorganism in the natural environment, nonobservance of hygiene rules during milk collection and mistakes in the technological process, and the ability of this microorganism to survive and multiply during food storage at refrigeration temperatures [Rola et al., 1994;Ganowiak, 1997; Rogga et al., 2005].

The aim of the present study was to assess the growth of *Listeria monocytogenes* in tvarog cheese during storage for 21 days in a wide range of temperature (3–15°C).

Materials and methods

Behaviour of *Listeria monocytogenes* was evaluated in tvarog, which was purchased from a local supermarket. Tvarog is a popular, traditional, polish, nonripening cheese, often used as a component of diet. It is produced from pasteurized cow's milk, as a result of milk fermentation, in many technological variants, with 35–42% fat content in dry mass, with 75% water content, pH about 4.5 and without addition of NaCl . This cheese is characterized by a mild, pure, slightly sour taste, compact, homogeneous texture, and white to slightly creamy color.

All materials used in this study were obtained from Merck (Warsaw, Poland), unless otherwise stated.

Bacterial culture

Strain *Listeria monocytogenes* 38 used in this study was obtained from culture collection of Chair of Industrial and Food Microbiology, Faculty of Food Sciences, University of Warmia and Mazury, Olsztyn. This strain was isolated from raw milk using Fraser I and II medium with selective supplements. Identification of *Listeria* strain was confirmed using fermentation tests (saccharides: rhamnose, saccharose, and xylose). Verification tests to define this strain to *Listeria monocytogenes* species were performed: CAMP (hemolytic) using strains *S. aureus* (ATCC 25923), *R. equi* (ATCC 6939), and model strains *L. monocytogenes* (ATCC 19112), *L. innocua* (ATCC 33090) and *L. ivanovii*. (ATCC 19119), and immunoenzymatic test Clearview- *Listeria* (Unipath Ltd., United Kingdom). Isolated and identified strain *L. monocytogenes* was registered in The Strain Collection of the Chair of Industrial and Food Microbiology, Faculty of Food Sciences, University of Warmia and Mazury, Olsztyn as *Listeria monocytogenes* 38.

Inoculum preparation

The strain was subcultured in Listeria enrichment broth (LEB, according to FDA) at 37°C for 18h and consecutive was maintained in LEB broth at 6°C. Cultures of *L. monocytogenes* 38 were transferred and incubated at 37°C for 18 h, before their use in experiment.

The initial level of *L. monocytogenes* 38 after transfer was about 4 x 10^8 cfu/mL. For each experiment bacterial suspension of microorganisms was serially diluted (1:10) in saline solution to concentration 4 x 10^4 /mL. The tvarog samples (approximately 25 g) were placed into the sterile stomacher bags (Interscience, BagMixer400, France) and inoculated to achieve the final level of 10^3 cfu/g.

On the day of experiment, sterile saline solution (225 mL) was added into the stomacher bags and samples were homogenized in the stomacher. From each of such prepared samples ten-fold dilutions were made and samples were surface plated on Oxford agar in four repetitions.

At the same time, control samples of tvarog (not contaminated with *Listeria monocytogenes*) were prepared and analyzed for absence of Listeria. All analyses were conducted in a vertical flow laminar chamber of Biohazard II (Esco, Class II Biohazard, Singapore).

Study organization

Seven samples of inoculated tvarog for each storage temperature (3, 6, 9, 12, and 15°C) were prepared. Analyses were conducted in the following days: 0 (immediately after inoculation),1, 2, 3, 4, 7, 11, 14, 17, and 21 of storage for each temperature. The measurement of the pH values into tvarog samples were determined on days 0 (immediately after inoculation), 4, 7, 11, 14, 17, and 21 using indicator papers (Whatman International Ltd, Maidstone, England). Samples were stored in the precise cooling incubators (Memmert GmbH, type ICP). The number of *L. monocytogenes* 38 was determined on Oxford agar with selective supplement (Oxford Listeria). The Oxford agar selective medium for isolation and identification of *L. monocytogenes* is in accord with the standard 143:1990 IDF-FIL for milk and dairy products.

Experiments were conducted three times on different samples of tvarog from the same producer in four replicates for each evaluated sample at different temperatures of storage.

All obtained data were fitted to Baranyi model using DMFit add-in and graphically compared with the generated prognoses in Growth Predictor (GP) software. Factors that simulate the behaviour of listeria in tvarog were introduced into GP as follows: initial level of *Listeria monocytogenes* (approximately 3 log CFU/g), lactic acid content (7900 ppm (0,79%)), water activity (0,98), and temperature identical with that used in studies. Determination of pH in tvarog during storage did not show any statistical significant changes, thus pH values in GP were established for 4.6 and 4,5 (for 3, 6 and 9,12,15 °C, respectively).

Time-temperature polynomial model (response surface) of the behaviour of *L. monocytogenes* 38 in stored tvarog, including the experimental data was created (second order polynomial) (Statistica 7.0, StatSoft Polska Sp. z o.o, Cracow , Poland).

Results and discussion

Present studies conducted on tvarog stored for 21 days at 3°C showed a decreasing tendency in number of *Listeria monocytogenes* 38 (Fig. 1 (DMFit)). The maximum concentration of *L. monocytogenes* 38 was found on day 0, immediately after inoculation (3.34 log cfu/g), whereas the lowest number of the cells was determined after 14 days of storage (2.66 log cfu/g).

Decrease in number of *Listeria monocytogenes* 38 in tvarog was observed also at 6°C. The level of *Listeria* on day 0 was 3.39 log CFU/g and deacresed to 2.35 log cfu/g on 17 day. (Fig. 2 (DMFit)).

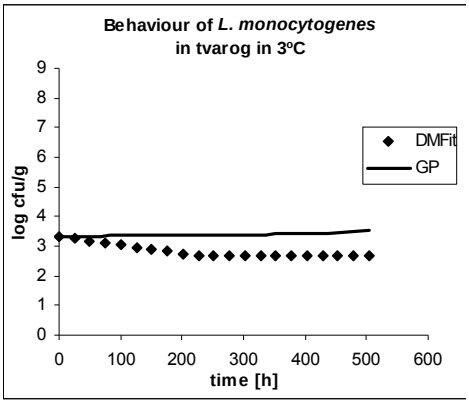

Fig. 1. Behaviour of *Listeria monocytogenes* in tvarog during storage for 21 days at temperture 3°C – DMFit – own studies, GP – predicted in computer software

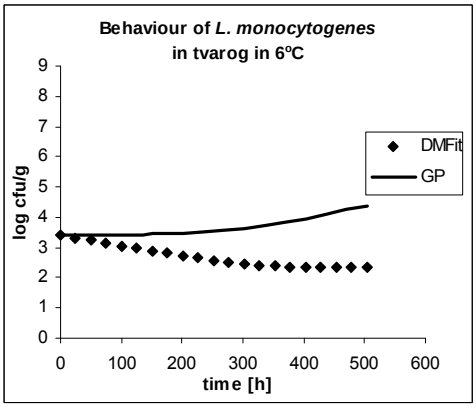

Fig. 2. Behaviour of *Listeria monocytogenes* in tvarog during storage for 21 days at temperture 6°C – DMFit – own studies, GP – predicted in computer software

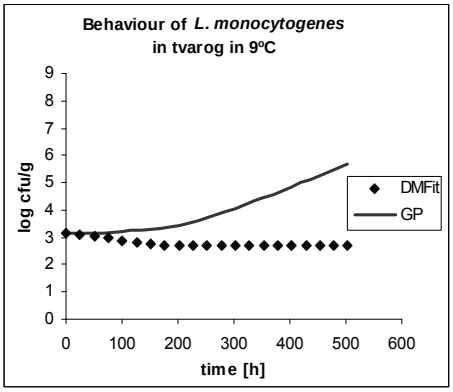

Fig. 3. Behaviour of *Listeria monocytogenes* in tvarog during storage for 21 days at temperture 9°C – DMFit – own studies, GP – predicted in computer software

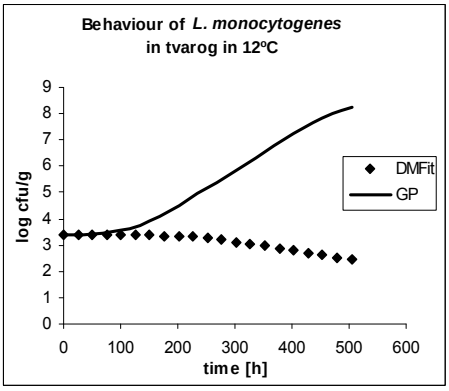

Fig. 4. Behaviour of *Listeria monocytogenes* in tvarog during storage for 21 days at temperture 12°C – DMFit – own studies, GP – predicted in computer software

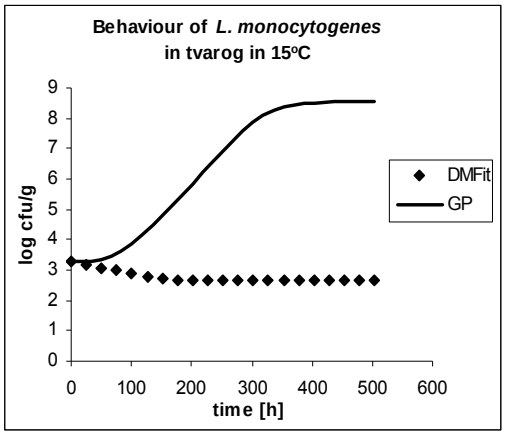

Fig. 5. Behaviour of *Listeria monocytogenes* in tvarog during storage for 21 days at temperture 15°C – DMFit – own studies, GP – predicted in computer software

In tvarog samples stored at 9°C insignificant decrease was found in the number of *Listeria* cells from 3.15 log cfu/g to 2.69 log cfu/g on 11 day. (Fig. 3 (DMFit)).

At 12°C, significant decrease in the number of bacterial cells was noticed from initial value of 3.37 log cfu/g to 2.43 log cfu/g after 21 days of storage (Fig. 4 (DMFit)).

Similar results were obtained for the samples stored at 15°C. In these tvarog samples *Listeria* population decreased from 3.27 log cfu/g to 2.36 log cfu/g after 11 days of storage (Fig. 5 (DMFit)).

It was found that the number of *Listeria* that survives in tvarog depends on the concentration of lactic acid [Steinka & Przybyłowski, 1997]. Tvarog cheese contains about 0.7% of lactic acid; thus, it is possible that this acid may decrease the number of these microorganisms. *Listeria* as a psychrotrophic pathogen tolerates refrigeration temperatures, so the low storage temperature cannot limit its growth. According to Ahamad and Marth [1989] the relationship between incubation temperature and inhibition of *L. monocytogenes* growth was particularly evident with lactic acid; the pathogen proliferated in the presence of 0.1% lactic acid at all temperatures except 7°C (incubation at temperatures from 7 to 35°C), 0.3 and 0.5% lactic acid led to only partial inactivation of the pathogen during extended incubation [Ahamad & Marth, 1989].

However, lactic acid bacteria, used as starter strains to produce variety of products, possess antagonistic properties in relation to other microorganisms. These properties result as production of numerous metabolites, such as lactic acid, acetic acid, aldehydes, diacetyl, hydrogen peroxide, and synthesis of specific antibiotics and bacteriocines. It was shown that *Lc. lactis ssp. lactis*, which is one of the components of the starter culture used for production of tvarog cheese, possesses the ability to synthesize a bacteriocin, nizin, which can inhibit Gram-positive bacteria [Koterska et al., 1998; Kot et al., 2000].

Our results on survival of *L. monocytogenes* in tvarog during storage at refrigeration temperatures are confirmed by studies conducted on Galotyri cheese (a traditional Greek soft cheese from buffalo milk) [Rogga et al., 2005]. Investigators determined behavior of *L. monocytogenes* in Galotyri cheese samples inoculated with the initial level 3 log CFU/g at

4°C for 28 days. Their results showed the maximum reduction in *Listeria* population during first days of storage (decrease by 1.6 log). This low level was maintained during next days of the experiment [Rogga et al., 2005].

Listeria monocytogenes was not found in all control samples.

Comparison growth of Listeria monocytogenes in tvarog in own studies and according to GP software

There are few publications concerning construction of predictive models on real food products. Studies are carried out mainly on microbiological culture media, especially on liquid. The created models are most often validated in relation to the specified food products during their production, storage and distribution [Kot et al. 2000; Pitt et al., 2000].

Therefore, it is suggested that such studies may not reflect the conditions in food products, because the growth is not limited by accompanying microflora and other food components.

Figures 1–5 show the behaviour of L. monocytogenes in tvarog in own studies (fitted into DMFit) and data obtained from simulation with GP software. Figures indicate that the growth of microorganisms in selected microbiological medium differs from their behaviour in the real food product.

log cfu/ml = 3,1881+0,0292*x-0,003*y-0,0015*x*x+2,8065E-5*x*y+2,6587E-6*y*y

R=0,79; R^2=0,6211; adj. R^2=0,6020; RSS=0,0345;Cp=6,0

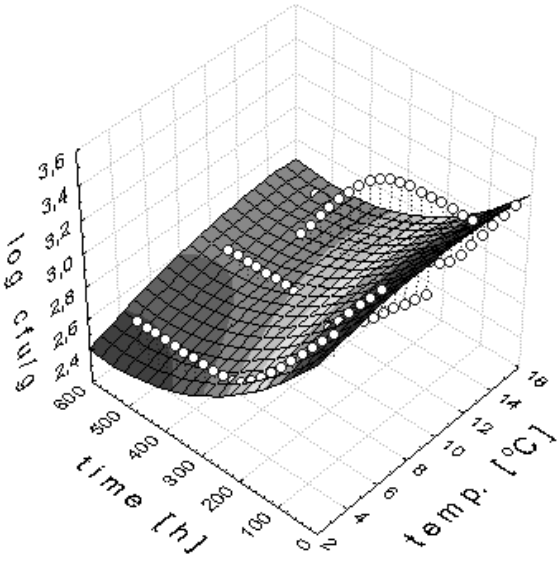

Fig. 6. Polynomial response surface model of *Listeria monocytogenes* in tvarog at the range of temperature 3–15°C during storage for 21 days

We found that the number of *L. monocytogenes* in tvarog decreased during storage and incubation temperature did not affect the number of *Listeria* cells.

Different results were obtained from GP software where, after characterizing environmental factors, growth of *Listeria monocytogenes* during 21 days was observed (from 3.34 to 3.51 and 3.27 log to 8.52 cfu/g, for 3 and 15°C, respectively). It is necessary to notice that GP takes into account lactic acid content, which can inhibit growth of this pathogen.

Moreover, the response surface model (Fig. 6) was constructed, describing the dynamics of studied microorganisms behaviour in tvarog as a function of microorganism number (log cfu/g; axis z), temperature (°C; axis x) and time (h; axis y). In order to determine the accuracy of this model to the experimental data the following statistical parameters were calculated: R = 0.78 (correlation coefficient), R^2 = 0.62 (coefficient of determination), adjusted R^2 = 0.60 (adjusted coefficient of determination), RSS = 0.0345 (residual sum of squares) and Cp = 6.0 (Mallows' statistical parameter). We found, that obtained values of statistical parameters are optimum for the second order polynomial [Stanisz, 2000].

Conclusions

It is assumed that the increase of storage temperature affects the activity of the lactic acid bacteria present in dairy products and the activity of substances produced by these microorganisms, which could negatively influence on *L. monocytogenes* cells.

Tvarog cheese is produced from pasteurized milk, in case of *L. monocytogenes* reinfection there is very little probability of an achievement and maintenance by this pathogen the population on the level (log 3cfu/g), that could threaten customer's health.

The present computer software, which simulates the pathogens' growth in various environmental conditions, did not consider many factors such as the availability of nutrients, antimicrobiological factors, or influence of the accompanying microflora. Therefore, many constructed in such way models cannot be adjusted to the results obtained from analyses of real food products [Pitt et al., 2000; Stańczak et al., 2000].

The results of this study confirm the thesis that it is necessary to conduct the microbiological analysis for the predictive model construction directly on the food product, and not on the liquid culture microbiological medium.

Models constructed in this way may be useful, for example, as a tool for control critical control points in the HACCP systems and for modification of recipes or new food products, before conducting expensive laboratory tests and pilot production.

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19

APPLICATION OF PREDICTIVE MICROBIOLOGY FOR MODELLING THE SAFETY OF MOZZARELLA CHEESE WITH REGARD TO *LISTERIA MONOCYTOGENES*

Introduction

Milk and other dairy products belong to highly perishable food products, and any faults in the technological process, or inappropriate quality of raw material, poses a risk of growth of foodborne pathogens. Listeria monocytogenes is one of the foodborne pathogens which is particularly important in terms of food safety issues [Giffel & Zwiettering, 1999; Valero et al., 2007]. This microorganism doesn't survive pasteurisation process, but as a result of postpasteurisation contamination or defective heat treatment, it can contaminate and multiply in food [Murphy et al., 1996]. According to the literature data, ca. 14.5% of cheeses in the retail may be contaminated with *Listeria monocytogenes* [Stecchini et al., 1995].

Listeria monocytogenes is a gram-positive, widely distributed in the natural environment microorganism, which demands special consideration cause it can proliferate during the refrigerated storage. The growth limits of *Listeria monocytogenes* were analysed in details and it is commonly assumed that *Listeria monocytogenes* grows in the temperature range from -1.5 to 45^{o}Cand pH 4.3–9.4 [Le Marc et al., 2002; Tienungoon et al., 2000]. There is a need for continuous occurrence monitoring of *Listeria monocytogenes* in food products, as this pathogen is responsible for many dangerous illnesses, and it poses particular risk for pregnant women and immunocompromised people.

Predictive microbiology, i.e. mathematical modelling of the growth or inactivation of microorganisms, is intensively developing subdiscipline of food microbiology. It is based on the following assumption: behaviour of microorganisms in a specified environmental conditions is reproducible; there's a possibility for modelling the behaviour of microorganisms on the basis of the microbiological observation made in past [Alavi et al., 1999; Black & Davidson, 2008].

Many mathematical models describing the influence of temperature and other factors on the kinetic parameters of the *Listeria monocytogenes* growth were generated. Most of them was created on the data obtained in microbiological media with defined chemical composition. Prognosis generated with such models, and their further validation, show significant errors in describing the behaviour of particular microorganism in food [Xanthiakos et al., 2006]. The differences result mostly from the number of factors that influence the growth of bacteria in foods, which we are not able to imitate in case of experiments in microbiological media.

The basic classification of predictive models divides them in 3 groups: primary, secondary and tertiary models. Primary models describe the behaviour of microorganisms under constant environmental conditions and calculate the basic growth parameters (i.e. growth

rate). The most frequently used primary model is that of Baranyi and Roberts [Baranyi & Roberts, 1994]. Secondary models analyse the influence of environmental conditions (temperature and others) on the primary models parameters. Commonly used model that belongs to that group is Ratkowsky model with all modifications. Tertiary models comprise of primary and secondary models. They are presented in the form of software's or other applications that simulate the behaviour of microorganisms. The most popular, in terms of accessibility, is Pathogen Modeling Program (www.ars.usda.gov) and ComBase Predictor (www.combase.cc). Pathogen Modelling Program was created as a research tool for assessing the effect of different factors on growth and survival of pathogens in food. It includes growth and inactivation models for many groups of microorganisms [Black & Davidson, 2008]. ComBase Predictor uses for modelling purposes the data generated in liquid microbiological media. Program utilizes the Baranyi and Roberts model as a primary model, secondary models are built on the basis of polynomial equations.

The aim of the following paper was: (i) evaluation the growth of *Listeria monocytogenes* in mozzarella cheese during the storage in temperature range 3–21°C, (ii) generation of the primary and secondary models describing the growth of *Listeria monocytogenes* in mozzarella cheese, and (iii) models validation in regard to growth data in different dairy products (www.combase.cc) and tertiary models simulations.

Material and methods

Listeria monocytogenes strain, mozzarella cheese inoculation and microbiological analyses.

Listeria monocytogenes strain used throughout the study was isolated from raw milk in the Chair of Industrial and Food Microbiology at the University of Warmia and Mazury in Olsztyn. The culture was maintained in LEB (Merck) and stored at 4°C. The inoculums was prepared from 24 h cultures in selective media LEB (Merck), incubated at 37°C for 18 h.

The research material was mozzarella cheese, which was bought in the retail store. Cheese was examined in terms of *Listeria monocytogenes* occurrence (Fraser, Merck). Cheese samples (25 g) were weighted and contaminated with appropriate dilution of *Listeria monocytogenes* culture to reach concentration ca. 10^3 cfu/g. Contaminated cheese samples were stored in highly precise incubators (Memmert) in the following temperatures: 3, 6, 9, 12, 15 and 21°C. Cheese samples were examined for the number of *Listeria monocytogenes* directly after inoculation and during storage; the frequency of sampling dependent on the storage temperature. Evaluation of the number of *Listeria monocytogenes* was performed with the traditional plate counts method (Oxford and ALOA, Merck). The experiment was performed in 3 replicates.

Predictive modelling

In order to generate primary models, the MS Excel add-in DMFit was used (J. Baranyi, Institute of Food Research, Norwich, UK). This software fits the Baranyi and Roberts model to experimental data according to equation:

$$N(t) = N_0 + \mu A(t) - \frac{1}{m} ln\left[1 + \frac{e^{m\mu A(t)-1}}{e^{mN_{\max}-N_0}}\right]$$

$$A(t) = t + \frac{1}{\mu} ln\left[\frac{e^{\mu(t)} + (e^{\lambda\mu - 1})^{-1}}{1 + (e^{\lambda\mu - 1})^{-1}} \right]$$

where t is the time (h), N_0 and N(t) is the microbial population in time 0 and t, μ is the growth rate (h^{-1}), λ is the lag phase duration (h), N_{max} is the maximum population density, m is the model parameter that characterises the transition from exponential to stationary phase [Baranyi & Roberts, 1994]. With the DMFit add-in, the growth rate μ was calculated, which was further used for secondary modelling according to Ratkowsy and Arrhenius equations.

Arrhenius model is based on the empirical equation, which analyses the relationship between the growth rate and storage temperature:

$$\mu = A \cdot e \frac{Ea}{RT}$$

where μ is the growth rate (h^{-1}), Ea is the activation energy (kJ/mol), R is the universal gas constant (8.31 $kJ \cdot mol^{-1} \cdot K^{-1}$), T is the absolute temperature (^oK). In case of proper fit of the equation to experimental data, a linear relationship will be obtained between lnμ and 1/T.

The Ratkowsky equation, or the square root type models, is the most often used secondary model:

$$\sqrt{\mu} = b(T - T_{\min})$$

where b is constant, T is the temperature (^oK), T_{min} is the minimum where μ = 0, and μ is the growth rate.

For tertiary modelling purposes, Pathogen Modeling Program and ComBase Predictor were used. Both application are available via the internet free of charge. Mutual property of this programs is the fact that they were constructed on the basis of experiments conducted in liquid microbiological media.

In order to check the predictive capacity of generated models, data from ComBase database concerning the growth of *Listeria monocytogenes* in dairy products (mainly in cheeses) were used. The selection criteria for choosing the appropriate microbiological data were as follows: (i) growth curves were generated in food products without addition of any other substances, (ii) in the temperature range 3–21^{o}C, (iii) the number of *Listeria* was determined with the traditional plate counts method.

Mathematical modelling is indispensably linked with model validation. In order to perform predictive models validation, graphical, statistical and mathematical methods are used. Common used factors describing the goodness-of-fit of models is bias B_f and accuracy A_f factors:

$$\sqrt{\left(\sum^{m} \frac{ln\left[f\left(x^{(k)} - ln\mu^{(k)}\right)^2 \right]}{m} \right)}$$

$$B_f = ((\Sigma_1(k=1)^{\uparrow} m\ (ln\ [(f(x^{\uparrow}((k)) - ln\ [(\mu^{\uparrow}((k)))]])\ /\ m)$$

Bias factor Bf ans wers the question: whether on average the observed values lie above or below the line of equivalence and, if so, by how much. It represents the general deviation of the model. Bias factor equals to 1 presents the perfect agreement between observed and predicted values [Baranyi et al., 1999; Ross, 1996].

Accuracy factor calculates the average distance between each observed point and the line of equivalence. It evaluates by how much the predictive values differ from the observed ones. The higher value of the accuracy factor, the worse accuracy of the prognosis [Baranyi et al., 1999; Ross, 1996].

Results and discussion

Growth of *Listeria monocytogenes* in mozzarella cheese during storage in the temperature range 3–21°C and fitted growth curves according to Baranyi and Roberts models shows Fig. 1. Applied model described the observed growth of bacteria in a satisfactory way (R^2>0.9).

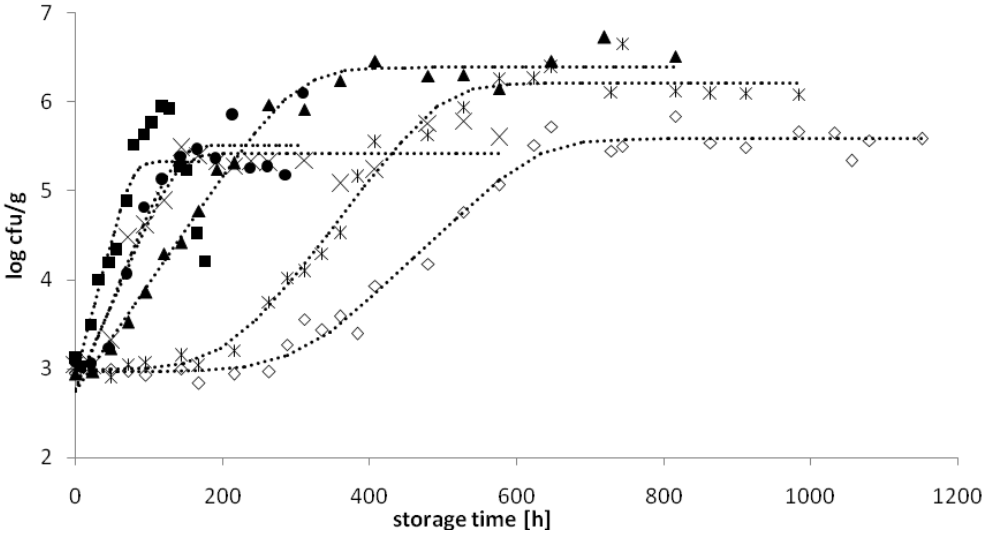

Fig. 1. Changes in the number of *Listeria monocytogenes* (log cfu/g) during storage in the following temperatures 3 (◊), 6 (∗), 9 (▲), 12 (x), 15 (●) and 21 (■)

Listeria monocytogenes proliferated in all applied temperatures, with the growth more dynamic as the incubation temperature increase. The duration of the lag phase, i.e. the adaptation phase of bacteria in the new environment, was highly correlated with the storage temperature and it contained in the range from 300 to almost 0 hours, at 3 and 21°C respectively. Obtained results are in accordance with the literature data, where the growth rate for *Listeria monocytogenes* in mozzarella cheese at 5°C was ca. 0.011h^{-1} [Dalgaard & Jorgensen, 1998].

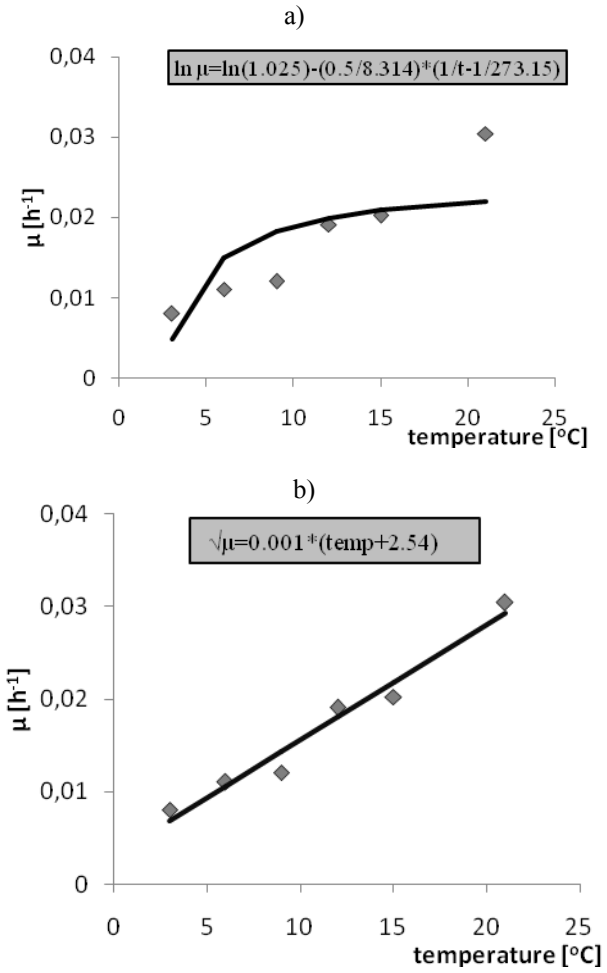

Fig. 2. Mathematical and graphical presentation of fitted models (a) Arrhenius and (b) Ratkowsky

During the secondary modelling the influence of storage temperature on the growth rate of *Listeria monocytogenes* in mozzarella cheese was analysed. Figure 2 represents mathematical models according to Ratkowsky and Arrhenius equations.

It can be visually stated that Ratkowsky model better describes analysed data than the Arrhenius model. It was also confirmed with mathematical validation. Observed growth rates μ_{obs} and the outcomes of secondary modeling, i.e. predicted growth rates μ_{pred} according to Arrhenius and Ratkowsky equations as well as the results of mathematical validation are presented in Table 1. The determination coefficients for Ratkowsky and Arrhenius models were 96.5 and 59.8% respectively. The estimated theoretical minimum temperature in the Ratkowsky equation was -2.53^{o}C. This value is in accordance with published minimum temperature for *Listeria monocytogenes* [Xanthiakos et al., 2006].

Table 1

Observed μ_{obs} and predicted μ_{pred} growth rates and results of mathematical validation

temp [°C]	Ratkowsky model				Arrhenius model			
	μ_{obs} [h^{-1}]	μ_{pred} [h-1]	B_f	A_f	μ_{obs} [h^{-1}]	μ_{pred} [h-1]	B_f	A_f
3	0.008	0.007	1.011	1.111	0.008	0.005	1.203	1.538
6	0.011	0.011			0.011	0.015		
9	0.012	0.014			0.012	0.018		
12	0.019	0.018			0.019	0.020		
15	0.020	0.022			0.020	0.021		
21	0.030	0.029			0.030	0.022		

According to bias and accuracy factors it was stated, that Ratkowsky model describes the relationship between the growth rate and temperature in more precise manner. It was confirmed by Figure 3, which shows the connection between observed and predicted growth rates, and presents the result of graphical validation. Stannard et al. analysed the relationship between temperature and growth rates of many microorganisms. They compared prognosis from Ratkowsky and Arrhenius models and concluded that the former gave better results [Griffiths, 1994].

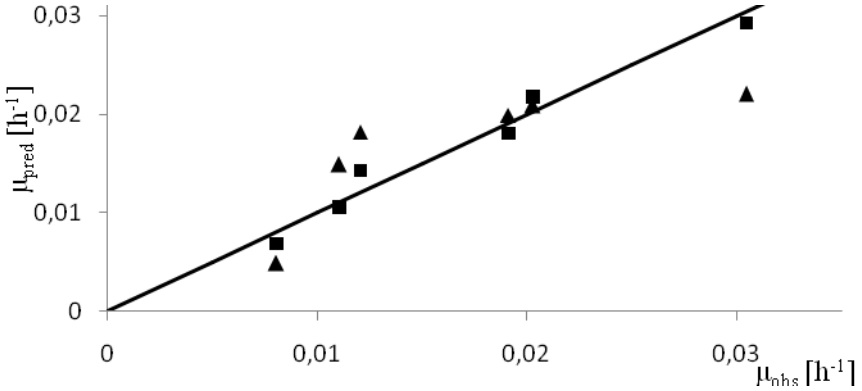

Fig. 3. Observed μ_{obs} and predicted μ_{pred} growth rates: ▲ –Arrhenius model, ■ - Ratkowsky model

The simulation performed in Pathogen Modeling Program (PMP) and ComBase Predictor (CM) didn't give satisfactorily results (Tab. 2).

Overestimation of the growth rate from the PMP and CP simulations results from the fact, that those applications generate the predictions on the basis of experimental data, obtained in the microbiological media, not in the real food products. This kind of overestimations are commonly in the literature [Dalgaard & Jorgensen, 1998; Murphy et al., 1996; Xanthiakos et al., 2006].

Table 2

Accuracy (A_f) and bias (B_f) factors which characterize the goodness-of-fit of the PMP and CP simulation to observed values

	PMP	CP
A_f	4.6	3.6
B_f	10.4	5.5

It is worth noticing that PMP gave much higher error than CP. The former application generates the simulations using the Gompertz equation, whereas the latter use the second order polynomials. In the literature that kind of situation occurs often, that PMP significantly overestimates growth of microorganisms [Giffet & Zwietering, 1999; Xanthiakos et al., 2006]. Giffel and Zwietering [1999] extracted 98 generation times of *Listeria monocytogenes* in dairy products in order to validate the predicted generation times of 3 predictive models, that were based on the experiments made in microbiological media. They concluded that analysed models considerably overestimate the growth of microorganisms, the accuracy and bias factors were 1.9–2.3 and 0.4–0.67 respectively.

The last purpose of the work was to check the predictive capability of generated Ratkowsky and Arrhenius models in terms of data from ComBase database, which containes answers of microorganisms under particular environment conditions [Baranyi & Tamplin, 2004]. Obtained results showed that models gave satisfactorily answer only in case when the analysed food product was mozzarella type cheese (A_f – 1.4-1.6; B_f – 1.1-1.25). Application of models to other dairy products was weighted with significant error. It confirms the hypothesis that predictive models, generated in order to increase food safety, must be based on the data conducted in food products, not in microbiological media.

Conclusions

In the following paper the growth ability of *Listeria monocytogenes* in mozzarella type cheese during storage in temperature range 3–21^{o}C was stated. It confirms the hypothesis that *Listeria monocytogenes* is able to reach high concentration in that kind of products, which poses a risk for consumers health. Good manufacturing practice and predictive modelling are needed in order to eliminate this dangerous situation. It was concluded that predictive models should be constructed on the basis of data generated in real foods, not microbiological media. Validation of predictive models dedicated for particular food product, or groups of products, showed relatively small fitting errors, what directly indicates the usefulness of predictive models.

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20

IDENTIFICATION OF GLUCOSINOLATES DETERMINED IN SEEDS, SPROUTS AND VEGETABLES FROM BRASSICACEAE FAMILY APPLYING LIQUID CHROMATOGRAPHY COUPLED WITH MASS SPECTROMETRY AND POSITIVE ELECTROSPRAY IONIZATION (LC-ESI/MS)

Introduction

Glucosinolates (GLS) are sulphur glucosides (Fig. 1) present in cruciferous plants as cabbage, cauliflower, Brussels sprouts, broccoli, turnip, radish and rape etc. It is said that occurrence of glucosinolates in food gives it functional and health promoting character. Glucosinolates after enzymatic, thermal or due intestinal microflora hydrolysis are precursors of a variety of degradation products like isothiocyantes, indoles, thiocyanates or nitriles [Fahey et al., 2001; Mikkelsen et al., 2002]. The glucosinolates' degradation products (GDP) are responsible for pungent and sour flavour of *Brassica* vegetables and moreover they have diverse structure, properties and biological activity. Certain GDP have antibacterial (e.g. towards *Helicobacter pylori*) and antifungal properties and several are well known from multidirectional anticarcinogenic potential [Fahey et al., 2001; Kyung & Fleming, 1994; Mikkelsen et al., 2002]. These phytochemicals may prevent tumorigenesis by stimulating apoptosis, inhibiting the enzyme activation of chemical carcinogens and modifying the steroid hormone metabolism [Bonnesen et al., 2001, Das et al., 2000; Higdon et al., 2007; Verhoeven et al., 1997]. Evidence from epidemiological studies and experiments on animals suggests that *Brassica* vegetables play an important role in the prevention of cancer of colon, rectum, thyroid, prostate, cervix, bladder and breasts [Higdon et al., 2007, Kristal & Lampe, 2002; Munday et al., 2008; Voorrips et al., 2000].

In the past, researchers were mainly focused on the negative aspects of glucosinolates, especially due their goitrogenic effects towards cattle or rabbits of defatted meal from traditional rapeseeds and some vegetables. Nowadays interest of glucosinolates covers not only its anticarcinogenic activities, but also the design of industrial and/or domestic processing to enhance the highest content and bioavability of GLS [Fahey et al., 2001, Vallejo et al., 2002].

The method for qualitative and quantitative analysis of glucosinolates (as desulfo- GLS) in rapeseeds is described in the international standard ISO 9167-1. There were several modifications applied by different authors to determine these phytochemicals as desulfo-compounds in *Brassica* vegetables. Samples preparation with purification of vegetables extracts and desulfation of glucosinolates on ion exchange columns increases complexity of the method, but also its selectivity. Taking into consideration that there are more than 120 glucosinolates known and only several are commercially available analysis using liquid

chromatography with spectrophotometric detection (HPLC–UV) causes a problem with identification. Application of diode array detector (DAD) gives additional information in the form of UV spectra of desulfoglucosinolates, however spectra can be identical for molecules with similar structure.

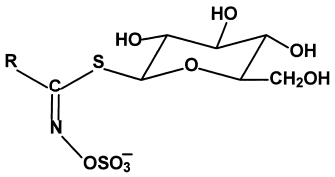

Fig. 1. Basic glucosinolate structure (R – variable side chain, amino acid derivative). Desulfoglucosinolate molecule has hydroxyl group instead of $-OSO_3^-$ group

The aim of this study was to elaborate the method of desulfoglucosinolates identification present in seeds, sprouts and vegetables from Brassicaceae family by means of liquid chromatography coupled with mass spectrometry (LC-MS).

Materials and methods

The analysis concerned seeds, sprouts and vegetables of broccoli (*Brassica oleracea* L. var *italica*), cauliflower (*Brassica oleracea* L. var. *botrytis*), red cabbage (*Brassica oleracea* L. var. *Capitata* f. *rubra*), radish (*Raphanus sativus* L.), and rapeseed (*Brassica napus* L. subsp. *napus*) and turnip rape (*Brassica rapa* subsp. *oleifera*). Material for the analysis was prepared according to the international standard ISO 9167-1, with some modifications [Sosińska & Obiedziński, 2007]. Glucotropaeolin or sinigrin were added as internal standard. Glucosinolates were extracted from frozen samples in liquid nitrogen with methanol (70ºC). Extracts were purified on ion exchange columns (Sephadex DEAE A25) and desulfation (sulfatase enzyme from *Helix pomatia*) was carried out. In the result desulfoglucosinolates (Fig. 1) in water solution were subjected to reversed phase liquid chromatographic resolution The analyses were conducted using liquid chromatograph coupled with mass spectrometer (with quadrupole filter) LCMS 2010 Shimadzu. Desulfoglucosinolates were injected onto Luna Phenomenex C18 RP column (150 mm x 4,6mm, 3µm), temperature was maintained at 35ºC. The mobile phase were water and acetonitrile (LCMS grade) employed with a flow rate 0,6 ml/min in gradient program. There was detection by photodiode array detector (DAD) conducted, moreover mass spectrometer with single quadrupole mass analyser and positive electrospray ionisation (ESI) interface was used. The outlet of the DAD detector was directly conducted to the ESI inferface. The conditions were set as follows: interface voltage 5 kV; heat block temp. 240ºC; CDL temp. 280ºC; CDL voltage 25 V; the spray was stabilised by a nebulizer gas, flow-rate (N_2) 1,2 L/min; detector voltage 1,5 kV; Q-array voltage DC 15,0 V, RF 125V; scan range: 200–500 m/z.

Results and discussion

Optimalization of electrospray ionization (ESI) conditions

There were different conditions tested to ensure desulfoglucosinolates efficient ionisation via electrospray in positive mode. Optimalizaton was conducted for desulfosinigrin (aliphatic GLS) and desulfoglucotropeaolin (aromatic GLS). Assayed ranges with optimal values, when the highest detector response was observed, are presented in Table 1. Those conditions were used for analysis of all identified desulfoglucosinolates in samples. There should be mentioned that addition of varied modifiers e.g. acetic acid (0,1–0,25%) or formic acid (0,1–0,25%) to the mobile phase was also tested without any insignificant improvement of ionisation efficiency.

Table 1

Assayed range and optimal values for condition of ESI-MS analysis of desulfoglucosinolates

Parameter	Unit	Assayed range	Optimum value
Interface voltage	kV	-5 ÷ 5	5
Heat block temperature	ºC	30 ÷ 300	240
CDL (curved desolvation line) temperature	ºC	30 ÷ 300	280
CDL (curved desolvation line) voltage	V	-150 ÷ 150	25
Gas flow-rate	L/min	0,5 ÷ 1,5	1,2

In the positive mode desulfoglucosinolates were detected as sodium $[M+Na]^+$ and potassium $[M+K]^+$ adducts, respectively 302 m/z and 318 m/z ions for desulfosinigrin (MW = 279); 352 m/z and 368 m/z ions for desulfoglucotropeaolin (MW = 329). In both cases sodium adduct was more abundant one, which can be explained as higher availability or/and affinity of sodium than potassium atoms to desulfoglucosinolate molecule (Fig. 2).

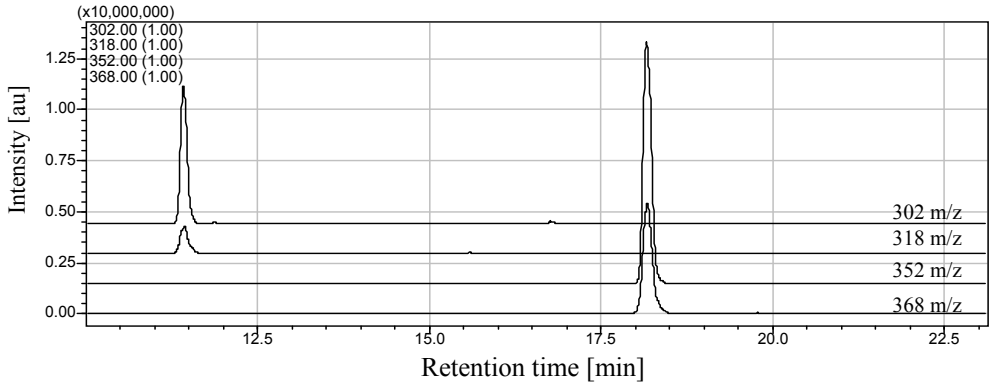

Fig. 2. ESI/MS chromatogram of desulfosinigrin (RT=11,40 min) and desulfoglucotropaeoiln (RT=18,15) in positive (b) mode

Identification of desulfoglucosinolates by ESI/MS in *Brassica* plants

On the basis of specific mass spectra eighteen desulfoglucosinolates were identified. In rape and turnip seeds the following glucosinolates were detected: progoitrin, epiprogoitrin, glucoalyssin, gluconapin, 4-hydroxyglucobrassicin, glucobrassicin and gluconasturtin. Whereas in seeds and spouts of broccoli and red cabbage progoitrin, sinigrin glucoraphanin, gluconapin, 4-hydroxyglucobrassicin, glucobrassicanapin, glucoerucin, glucobrassicin, gluconasturtin, 4-metoxyglucobrassicin and neoglucobrassicin were identified. Moreover in seeds and sprouts of small radish not only progoitrin, 4-hydroxyglucobrassicin and 4-metoxyglucobrassicin were detected, but also glucoraphenin and dehydroerucin. In vegetables like broccoli and cauliflower glucoiberin, progoitrin, sinigrin glucoraphanin, 4-hydroxyglucobrassicin, glucobrassicanapin, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin were detected.

Sodium $[M+Na]^+$ and potassium $[M+K]^+$ adducts of all identified desulfoglucosinolates were detected. Mainly the most abundant adduct was the sodium one, and the potassium has relative abundance in range 14% in desulfosinigrin mass spectrum and 90% in desulfoglucobrasscin (Table 2). Only in case of desulfo-4-methoxyglucobrassicin and desulfoneoglucobrassicin the potassium adducts have 100% relative abundance, the sodium one has 64% and 53%, respectively. Creation of this two adducts for each desulfoglucosinolate provides additional confirmation, esspecially when a single quadropole analyser is used. There were also detected $[M\text{-glucosyl}]^+$ ions, in case of desulfoglucobrassicin (207 m/z), desulfoneoglucobrassicin (237 m/z), desulfo-4-methoxyglucobrassicin (237 m/z), desulfo-4-hydroksyglucobrassicin (223 m/z) and desulfoglucoalyssin (210 m/z). Similary, Vallejo et al. [Vallejo et al., 2002] has detected the presence of $[M+Na]^+$ and M-glucosyl ions in case of indolyl glucosinolates mentioned above. Example ESI/MS spectra of desulfoglucoraphanin is shown on Figure 3.

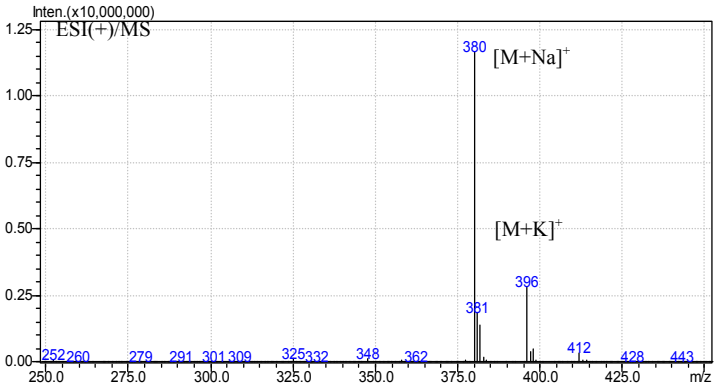

Fig. 3. ESI/MS spectra of desulfoglucoraphanin in negative and positive mode

It is must be taken into consideration that the electrospray ionisation and detection with single mass spectrometer provide the identical mass spectra for desulfo-4-metoxyglucobrasscin and desulfoneoglucobrasscin. The identification of these compounds was based on different retention times: 20,73 and 25,94 min respectively.

Table 2

Desulfoglucosinolates identified in *Brassica* plants, with nominal molecular weight and most abundant ions in spectra

No.	Ret. time	Common name / chemical name of side chain	Molecular weight	Positive mode (relative abundance [%])	
				$[M+Na]^+$	$[M+K]^+$
1	9,75	desulfoglucoiberin / 3-(methylsulfinyl)propyl	343	366 (100)	382 (33)
2	10,46	desulfoprogoitrin / 2(R)-hydroxy-3-butenyl	309	332 (100)	348 (21)
2	10,62	desulfoepiprogoitrin / 2(S)-hydroxy-3-butenyl	309	332 (100)	348 (20)
3	10,75	desulfoglucoraphanin / 4-(methylsulfinyl)butyl	357	380 (100)	396 (47)
4	11,08	desulfoglucoraphenin / 4-methylsulfinyl-3-butenyl	355	378 (100)	394 (32)
5	11,37	desulfosinigrin / 2-propenyl	279	302 (100)	318 (14)
6	12,14	desulfoglucoalyssin / 5-(methylsulfinyl)pentyl	371	394 (100)	410 (35)
7	13,54	desulfoglucoibarin / 7-(methylsulfinyl)heptyl	385	408 (100)	424 (39)
8	14,35	desulfogluconapin / 3-butenyl	293	316 (100)	332 (20)
9	15,14	desulfo-4-hydroksyglucobrassicn / 4-hydroxyindol-3-ylmethyl	384	407 (100)	423 (73)
10	15,47	desulfoglucoiberverin / 3-(methylthio)propyl	327	350 (100)	366 (53)
11	16,97	desulfoglucobrassicanapin / 4-pentenyl	307	330 (100)	346 (52)
12	17,87	desulfoglucotropaeolin / benzyl	329	352 (100)	368 (31)
13	17,97	desulfoglucoerucin / 4-(methylthio)butyl	341	364 (100)	380 (60)
14	18,80	desulfodehydroglucoerucin / 4-methylthio-3-butenyl	339	362 (100)	378 (84)
15	19,21	desulfoglucobrasscin / indol-3-ylmethyl	368	391 (100)	407 (90)
16	20,73	desulfo-4-methoxyglucobrassicin / 4-methoxyindol-3-ylmethyl	398	421 (64)	437(100)
17	21,33	desulfogluconasturtin / 2-phenylethyl	343	366 (100)	382 (71)
18	25,94	desulfoneoglucobrassicin / 1-methoxyindol-3-ylmethyl	398	421 (53)	437(100)

In the past methods for the glucosinolates' analysis, as intact and desulfoglucosinolates, involved mainly reversed-phase HPLC with spectrophotometric detection [Brown et al., 2003; Hrnčiřik et al., 1998; ISO 9167-1:1992; Kaushik & Agnihotri, 1999; Padilla et al., 2007]. Later different methods using mass spectrometric detection were applied. Liquid chromatography coupled with mass spectrometry (LC–MS) methods can be used to identify glucosinolates using atmospheric pressure chemical ionisation (APCI) [Bennett et al., 2002; Griffiths et al., 2000; Kim et al., 2001; Tolra et al., 2000] or electrospray ionisation (ESI) [Bennett et al., 2002;Mellon et al., 2002; Zrybko et al., 1997]. Glucosinolates can be identified by negative ion electrospray LC–MS/MS with MRM (multiple reaction monitoring) fragmentation and SRM (Selected reaction monitoring) detection in case of intact glucosinolates [Skutlarek et al., 2004; Song et al., 2005; Tian et al., 2005] or neutral loss method applied in case of desulfated ones [Matthaus & Luftmann, 2000]. The next issue is the possibility to obtain different ions during glucosinolates analysis. Matthaus & Luftmann [Matthaus & Luftmann, 2000] determined $[M+H]^+$ ions of several desulfoglucosinolates using the electrospray ionisation, likewise Griffiths et al. [2000] using atmospheric pressure chemical ionisation. Beyond protonated desulfoglucosinolates molecues Bennett et al. [2002], has analyzed sodium and potassium adducts created during the atmospheric pressure chemical ionization. Vallejo et al. [2002] has also observed the occurrence of adducts $[M+Na]^+$ and other fragments like M-glucosyl, M-glucosyl-methoxy and some not identified after elecrospray ionisation.

Conclusions

The individual desulfoglucosinolates present in selected cruciferous plants were detected and identified using liquid chromatography coupled with mass spectrometry, with positive electrospray ionisation applied (LC-ESI/MS). The optimal conditions for positive electrospray ionisation were settled. Desulfoglucosinolates were detected as sodium $[M+Na]^+$ and potassium $[M+K]^+$ adducts in the postivie mode ESI(+).

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