NEW TRENDS

IN FOOD ANALYTICS

edited by
Marek Szołtysik
and Anna Dąbrowska

NEW TRENDS
IN FOOD ANALYTICS

Wrocław 2011

Authors:

Biernat Jadwiga, Bodkowski Robert, Bulla Jozef, Buszewski Bogusław, Chmiel Marta, Dasiewicz Krzysztof, Dobrzański Zbigniew, Filipiak Marian, Florowski Tomasz, Goldsztejn Magdalena, Golian Jozef, Grajewa Halina, Grenda Tomasz, Gwiazdowska Daniela, Jamroz Dorota, Jasiczak Jan, Kikut-Ligaj Dariusz, Kowalik Jarosław, Kukier Elżbieta, Kwiatek Krzysztof, Ligaj Marta, Ligor Magdalena, Lochyński Stanisław, Łobacz Adriana, Marek Szołtysik, Maršálková Lenka, Maruška Audrius, Nawracała Jerzy, Nowakowski Piotr, Nuc Katarzyna, Patkowska-Sokoła Bożena, Perkowski Juliusz, Pękala Jolanta, Pieczyńska Joanna, Skolik Agnieszka, Słowiński Mirosław, Steininger Mieczysław, Stuper Kinga, Tichoniuk Mariusz, Trziszka Tadeusz, Ziajka Stefan, Židek Radoslav

Editors:

Marek Szołtysik

Anna Dąbrowska

Reviewers:

Józefa Chrzanowska, Tadeusz Trziszka, Wiesław Kopeć, Marek Szołtysik, Anna Dąbrowska, Małgorzata Korzeniowska, Łukasz Bobak, Anna Zimoch

Editorial correction

Elżbieta Winiarska-Grabosz

Editorial violations

Adam Broda

Cover design

Kornel Owczarek

Monography CXXIV

ISSN 1898–1151
ISBN 978-83-7717-062-5

WYDAWNICTWO UNIWERSYTETU PRZYRODNICZEGO WE WROCŁAWIU
Redaktor Naczelny – prof. dr hab. Andrzej Kotecki
ul. Sopocka 23, 50–344 Wrocław, tel. 71 328–12–77
e-mail: wyd@up.wroc.pl

Nakład 150 + 16 egz. Ark. wyd. 8,7. Ark. druk. 8,5
Druk i oprawa: Elma

CONTENTS

FOREWORD

The dynamic development of food science and progressive globalization of food markets have required the use of various modern methods of food analysis. These methods focus mainly on the examination of basic quality parameters in the production of raw materials and finished products. They also involve fast inter-operational control of technological processes, determination of changes in food during storage, and the identification of numerous misstatements.

The common distinction between classical and instrumental analysis is becoming increasingly inadequate, as the current advancement of material engineering and information technology have resulted in many traditional methods being automated and transferred to analytical equipment designed to perform serial determinations. It is difficult to perform a clear classification of many recently developed methods in terms of agents used or method of detection. Analyses combining physical, chemical, biochemical, imunoenzymatic and other factors are no longer surprising, and are increasingly common in standard food tests.

The progress observed in food analysis mainly consists in the modifications of traditional methods and their automatization. Another important issue is the search for new alternative ways of testing food and the miniaturization and simplification of techniques. A key element is the speed and reliability of detection of a given analyte in different matrices. This is particularly important in safety management systems, such as GMP/GHP, HACCP and ISO 22000. Their use in the food industry is intended to eliminate health risks in the short term, which in turn necessitates the development of control systems requiring fast methods of food testing.

Modern food analysis also requires the implementation and application of quality management systems. The aim is to obtain reproducible results which reflect real situations. Therefore the introduction of many activities based on good laboratory practice (GLP) or the requirements of ISO 170025, "General requirements for the competence of testing and calibration laboratories" are becoming standard for many food analysis laboratories. The idea of implementing quality management systems in the laboratory is dictated by the need to ensure mutual trust in relations between the laboratories and clients, and also the necessity of using analytical standards that make it possible to limit multiple testing of food products and to facilitate the free movement of food products in the world markets.

This monograph presents many practical and contemporary issues in the area of food analysis. It contains interesting examples of the preparation of samples, separation techniques such as liquid and gas chromatography, as well as methods of microbiological analysis using molecular biology tools. These issues and examples are well complemented by chapters on quality management in analytical laboratories. Although this study is not a complete compendium of knowledge in the field of food analysis, it is a great tool for expanding knowledge and improving the skills of the operators of any modern analytical laboratory.

Marek Szołtysik

Anna Dąbrowska

1

MOLECULAR METHODS FOR *AEROMONAS HYDROPHILA* DETECTION

Introduction

In epidemiological studies, referring to the WHO report, the opportunistic pathogens, belonging to the species of *Aeromonas*, *Pseudomonas* and *Mycobacterium*, are detected much more from patients, than commonly recognized as dangerous, the bacteria *Vibrio*, *Campylobacter* and *Salmonella* [Morgan et al. 1985, Martin-Carnahan and Joseph 2005, Naharro et al. 2009].

Aeromonas hydrophila, the species of bacteria of the genus *Aeromonas*, receiv increasing attention because of its association with human disease and foodborne infections [Palumbo et al. 1989, Seshadri et al. 2006, Di Bari et al. 2007, Naharro et al. 2009]. This bacterium is included on the Contaminant Candidate List of the U.S. Environmental Protection Agency as a potential waterborne pathogen and was isolated from freshwater and variety of foods, like vegetables, raw milk, meat and dairy products, also stored in refrigerating conditions. *Aeromonas hydrophila* is associated with both diarrheal and extraintestinal infections in human disease, especially dangerous for children and persons with impaired immune system [Arnheim and Erlich 1992, Galindo and Chopra 2007]. The symptoms of the pathological features associated with infection caused by *A. hydrophila* refers to local enema, tissue necrosis, sepsis and mortality [Ljungh and Wadstrom 1983], resulting in the occurrence of disease entities such as gastroenteritis, wound infections, septicaemia, meningitis, peritonitis, endocarditis, osteomyelitis, etc. [Vally et al. 2004] and septicemia [Figueras 2005, Galindo et al. 2006; Janda and Abbott 1998]. The first documented human infection caused by *A. hydrophila* occurred in 1954 [Naharro et al. 2009]. Elevated numbers of *Aeromonas* spp. is correlated with natural disasters, for example in floodwaters after hurricane Katrina in New Orleans [Presley et al. 2006] and tsunami in southern Thailand in 2004 [Hiransuthikul et al. 2005].

Detection of *Aeromonas hydrophila* by conventional methods is complicated and time consuming. For rapid *A. hydrophila* detection we developed the molecular biology methods that were used for specific DNA fragments identification. The key component in determining of potential pathogenicity of this bacterium is aerolysin (aerA) gene, basic hemolysin produced by *A. hydrophila*. For the detection of this virulence factor a polymerase chain reaction (PCR) was used. To verify an expression of aerA gene in *A. hydrophila* cells the reverse transcriptase-polymerase chain reaction (RT-PCR) was applied. Confirmation that the PCR product is aerA gene fragment relied on the application of radioactively labeled DNA probe in Southern blotting method. The probe was also utilized for construction of electrochemical DNA biosensor for aerA gene fragment detection.

Aerolysin, toxin produced by *Aeromonas hydrophila*

The majority of human isolates of *Aeromonas hydrophila* are hemolytic. Characteristic for extraintestinal infections is soft tissue necrosis. That suggest than hemolysin is a virulence factor of *A. hydrophila*. These bacteria produced several extracellular toxins which may be responsible for the pathogenesis [Asao et al. 1984, Burke et al. 1981, Johnson and Lior 1981, Rose et al. 1989]. But the greatest importance is given to aerolysin, cytolytic enterotoxin first described by Bernheimer and Avigad [1974]. This extracellular toxin is produced by pathogenic strains of *A. hydrophila* [Asao et al. 1984, Seshadri et al. 2006, Naharro et al. 2009]. Aerolysin is soluble, thermolabile single protein molecule with a molecular weight of about 49–52 kDa, that exhibit both hemolytic and cytolytic properties [Seshadri et al. 2006, Naharro et al. 2009]. Toxin binds to specific glycoprotein receptors on the surface of eucaryotic cells, penetrates into the lipid bilayer and forms holes diameter of 1.5 nm, which results in leakage of cytoplasm [Seshadri et al. 2006, Galindo and Chopra 2007]. Studies of its toxicity in animals have shown high mortality of mice and rats. Lethal dose for 50% of the population of mice was determined at 27.5 ng [Johnson and Lior 1981, Ferguson et al. 1997, Naharro et al. 2009]. As suggested by Chakraborty et al. [1986], the gene encoding aerolysin is designated as aerA. In 1987 the sequence of 2 346 base pairs (bp) of the aerolysin gene from *Aeromonas hydrophila* was published by Howard et al. [1987]. The longest open reading frame extends from 532 to 1989 bp. This would produce a protein with a molecular weight of 53 800 Da. The translation product of the first 69 bp beginning at 532 is a typical 23-aminoacids signal peptide. Other regions of the gene coding promoter, palindromic and regulatory sequences.

Molecular methods for the detection of aerolysin gene

The detection of virulence factors as aerolysin is a key component in determining of pathogenic strains of *Aeromonas hydrophila* [Sen and Rodgers 2004]. Molecular methods enabled detection of specific DNA sequences may be used for aerolysin gene identification in genetic material samples. For this purpose mainly PCR techniques with the use of specific primers are proposed. Also other methods, as presented in this work Southern blot analysis and DNA biosensors devices, based on hybridization reaction may be useful.

PCR techniques

Polymerase chain reaction (PCR) is an enzymatic process of amplification of specific sequences of DNA. Is a mapping of the DNA replication process taking place in living cells. Theoretically, one DNA molecule after 30. cycles of PCR, lasting a total of just over an hour, can be amplified to 1 073 741 824 copies [Arnheim and Erlich 1992, Napierala et al. 2004]. Amplified DNA fragment with a specific length may be observed on the gel after electrophoresis. For this reason, PCR has revolutionized the area of diagnostic tests, which are also of crucial importance in the detection of pathogenic bacteria.

The first scientific work on the detection of aerolysin gene from *A. hydrophila* by PCR was published in 1990 [Pollard et al. 1990]. In the next years there have been subsequent others publications on the application of PCR to detection of this virulence factor [González--Serrano et al. 2002, Wang et al. 2003, Singh et al. 2009, Yogananath et. al 2009]. Table 1 shows sequences of primers used in PCR to identify of pathogenic strains of *A. hydrophila* on the base of aerolysin gene detection.

Table 1

Base sequences and predicted sizes of PCR products for the published of aerolysin gene specific primers

Primer	Oligonucleotide sequence (5'- 3')	Size of PCR product (bp)	Reference
1F 2R	GCAGAACCCATCTATCCAG TTTCTCCGGTAACAGGATTG	254	González-Serrano et al. 2002, Arora et al. 2006
Aerola Aerolb	CCAAGGGGTCTGTGGCGACA TTTCACCGGTAACAGGATTG	209	Pollard et al. 1990
AHF8 AHR8	ATGAAAAACAAAAAACCACG CAAATTCA TCACCCGTCTACGCTTGTCGGTA	1 417	Singh et al. 2009
Aer 2F Aer 2R	AGCGGCAGAGCCCGTCTATCCA AGTTGGTGGCGGTGTCGTAGCG	416	Yogananath et al. 2009
aerAF aerAR	CAAGAACAAGTTCAAGTGGCCA ACGAAGGTGTGGTTCCAGT	309	Wang et al. 2003

Hybridization methods

Nucleic acid hybridization has become one of the most important techniques in molecular biology for the detection and analysis of specific DNA sequences. In this method the specific DNA probes were used. The probes are complementary to the target single stranded DNA sequence and after base pairing double stranded DNA was formed. Molecular probes are used in various techniques in molecular biology. In this work the Southern blotting and DNA biosensor were presented.

For Southern blot analysis DNA isolated from organisms or amplified with specific primers was separated by gel electrophoresis and transferred from agarose gel onto a membrane which is then incubated with a DNA probe. The probe will form base pairs with its complementary DNA

sequence (target) and form a double stranded DNA. The probe is labelled before hybridization, for example with the use of radioactive or fluorescent molecules. Hybridization is then detected by visualizing the hybridized probe via autoradiography or chemiluminescent techniques.

DNA biosensors selectively detecting DNA sequences by hybridization appear very promising analytical tool [Hall 2002]. DNA hybridization biosensor consists of a biological recognition element associated with a transducer translating recognition event into a physically measurable value [Pividori et al. 2000]. Methods of signal transduction include mostly electrochemical, piezoelectric or optical systems. Electrochemical transducers are relatively simple devices, rather cheap, appropriately sensitive and selective, and generally eligible for routine tests, therefore they seem to be particularly attractive for the detection of specific DNA sequences [Pividori et al. 2000, de-los-Santos-Álvarez et al. 2004]. In an electrochemical DNA hybridization sensor usually a short single-stranded DNA probe is immobilized on working electrode to create recognition element. Among variety of working electrodes carbon paste electrodes (CPE) have been widely used to assemble DNA biosensor [Pividori et al. 2000]. The probe immobilized on the electrode surface is able to form double-stranded hybrid with its complementary nucleic acid (target). The hybridization event is commonly detected with the use of electroactive redox indicator that enables discrimination between single-stranded and newly formed on the electrode surface double-stranded DNA [Wang 1999, Pividori et al. 2000, de-los-Santos-Álvarez et al. 2004]. In this analytical approach bipyridyl or phenanthroline metal chelates (Ru, Os, Co), daunomycin, methylene blue are frequently used.

Application of the molecular methods for the detection of aerolysin gene

PCR assay

The aerolysin-specific oligonucleotide primers used in PCR were designed by computer analysis by using sequences of the aerA gene published in NCBI (National Center of Biotechnology Information) gene bank. The specifity of primers was checked by the nucleotide/nucleotide BLAST algorithm.

The PCR assay was carried out in 0.5 ml micro centrifuge tubes, with 25 µl of reaction mixture consisting of 15.3 µl sterile distilled water, 2.5 µl 10 x PCR buffer, 1.5 µl 25 mM $MgCl_2$, 1 µl 5 mM dNTP, 0.5 µl of 2.5 µM primers, 1 U of Taq DNA polymerase and 40 ng of DNA template (DNA was extracted from a reference strain of *Aeromonas hydrophila* ATCC 7966 with the use of DNA Genomic Maxi AX kit, A&A Biotechnology, Poland). PCR reagents were purchased from Novazym, Poland. The primers were synthesized by Tib Molbiol, Poznan, Poland. The amplification procedure consisted of an initial denaturation step at 94°C/2 min followed by 30 cycles with denaturation at 94°C/25 s, annealing at temperature gradient from 51.5 to 65.5°C/30 s and extension at 72°C/30 s. A final extension step was done at 72°C/2 min. For the detection and confirmation of the PCR products the gel electrophoresis were used. As a size marker a Nova 100bp DNA ladder, consists of 11 fragments ranging in *length* from 100 to 1500 bp, was used. The amplified DNA fragments were visualized by UV fluorescence after being stained with SYBR GOLD dye.

The forward primer at the sequence 5'CTGCGAGGGTTATCGTTGTG and the reverse primer at the sequence 5'GTGTCGCTGTCGTTGATCG amplified a 156 bp fragment of aerA gene (Fig. 1).

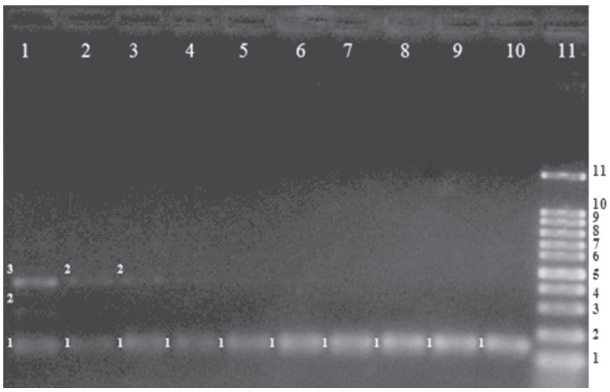


Fig. 1. Gel electrophoresis of PCR products in temperature gradient from 51.5 to 65.5°C (lanes 1–10). Lane 11 – Nova 100bp DNA ladder

Optimal temperature of primers annealing to DNA template should be experimentally tested using gradient PCR from 51.5 to 65.5°C. Estimated optimal annealing temperature was 64.6°C (lane 9 on Fig. 1) and from 57.6 to 65.5°C was achieved target PCR product without nonspecific DNA fragments.

For confirmation of aerA gene expression in *A. hydrophila* cells the reverse transcriptase-polymerase chain reaction (RT-PCR) was applied. RNA was isolated from bacteria with the use of the Total RNA Mini kit (A&A Biotechnology, Poland). In RT-PCR protocols RNA was first transcribed into cDNA by reverse transcriptase using VerteKIT (Novazym). The resulting cDNA (2.5 µl) was then used as template for PCR amplification at establishment optimal parameters with the use of primers specific for aerolysin gene. As positive control for RT-PCR were transcribed and amplified two housekeeping genes: gyrB encoding the B subunit of DNA gyrase (with the use of primers: 5'GATCTCTTCCCAGGAGGTGG and 5'CTCTTCCCAGGAGGTGGG with PCR product of 337 bp) and rpoD encoding the sigma subunit of RNA polymerase (with the use of primers: 5'AACCAGGTACAGAGTTCCGTC and 5'TACTCGAACCAGGCAGTTTCG with PCR product of 584 bp). As negative control, RT-PCR was conducted without of cDNA template, with the addition of all pairs of primers.

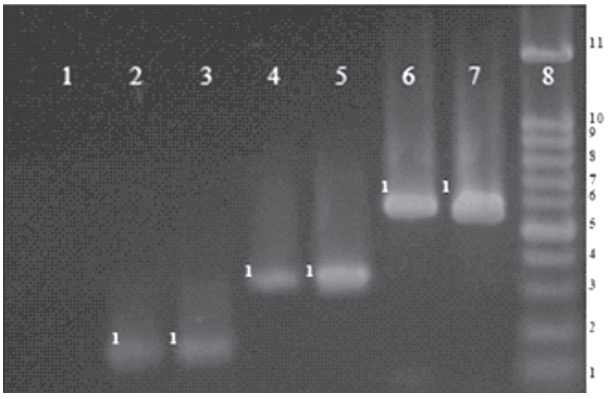


Fig. 2. Gel electrophoresis of RT-PCR products. aerA gene fragment (lanes: 2, 3), positive control for RT-PCR (gyrB gene: lanes 4, 5 and rpoD gene: lanes 6, 7) and a negative control (lane 1). Lane 8 – Nova 100bp DNA ladder

The results of RT-PCR shown in Figure 2 confirm the expression of aerA gene in the cells of *A. hydrophila*. As can be seen that aerA gene expression is slightly lower compared to housekeeping genes.

For the confirmation of specific aerA gene fragment in amplified PCR product the hybridization with DNA probe was applied. Aerolysin-specific nucleotide sequence of probe was found after searching of gene bank with the use of nucleotide/nucleotide BLAST algorithm. The designed probe at the sequence 5'GTCAAGACGGTGGTGGGCTG was used in Southern blot technique and for construction of electrochemical DNA biosensor.

Southern blotting

The amplified fragment of aerolysin gene was analyzed by electrophoresis through a 1% agarose gel. DNA was treated with alkaline solution containing sodium hydroxide for 30 minutes to denature DNA and obtain single strands. The denaturated DNA fragment was transferred to a nitrocellulose filter, which was next air dried and baked between two sheets of Whatman No. 3MM paper for 2 hours at 80°C. The membrane was then treated with biotin-labelled probe. Following hybridization the filter was washed three times in 5xSSCP and once in 1xSSCP. Detection of hybridization results was performed using Phototope Star Detection Kit, based on chemiluminescence, according to the manufacturer's instructions (Biolab, New England).

Fig. 3. Southern-blot hybridization of PCR amplified aerolysin gene fragment with biotin-labelled probe. There was an amplified DNA fragment in all lanes

The obtained results presented on the Figure 3 confirmed that in amplified DNA fragment the nucleobase sequence complementary to the probe was detected. This evidence, that this is the aerolysin gene.

DNA biosensor

Aerolysin gene specific DNA sensor was developed by the covalent attachment of designed DNA probe to carbon paste electrode modified by stearic acid and ethylenediamine

according to our procedure described previously [M. Ligaj et al. 2006]. For hybridization a probe modified electrode was immersed for 20 min in buffer containing a PCR amplified DNA sample. Then the electrode was washed and rinsed for 5 min to interaction with hybridization indicator (38.5 μM $Co(bpy)_3^{3+}$). After that the electrode was washed and finally a square wave voltammetric scan was recorded. The same reaction was performed with the use of noncomplementary DNA fragments (356 bp of 16S rRNA fragment amplified with the use of primers: 5'GGGAGTGCCTTCGGGAATCAGA and 5'TCACCGCAACATTCTGATTTG) in PCR.

Electrochemical measurements were performed with a potentiostat PGSTAT12 with GPES software (Eco Chemie, Utrecht, The Netherlands). The experimental conditions for electrochemical analysis were: the three electrode system consisted of a carbon paste working electrode, Ag/AgCl reference electrode and a platinum wire counter electrode. The carbon paste was prepared by mixing graphite powder with mineral oil with the ratio 70:30, the outer layer of electrode was prepared from 5% (w/w) stearic acid modified carbon paste. Experimental conditions for square wave voltammetry were: frequency of 50 Hz, amplitude of 0.04 V and step potential of 0.015 V.

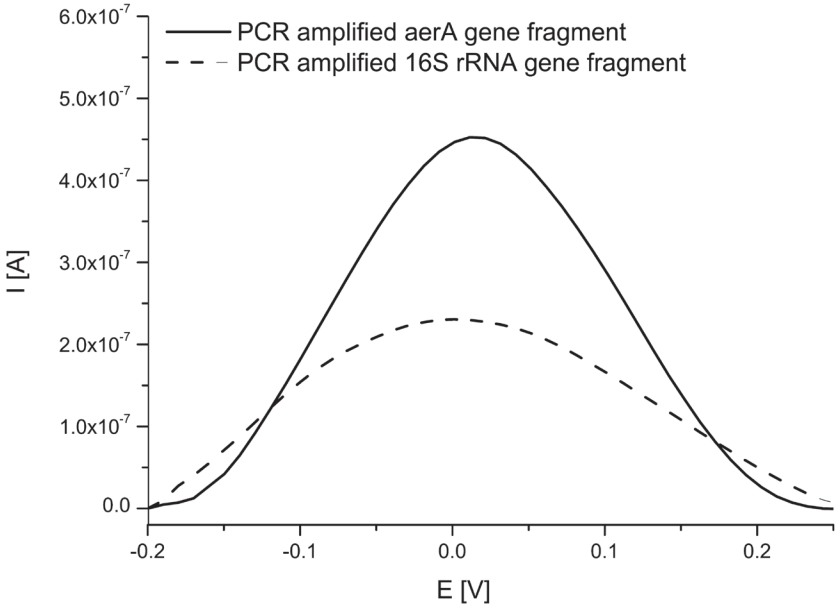


Fig. 4. SWVs of 38.5 μM $Co(bpy)_3^{3+}$ obtained after hybridization with PCR amplified aerA gene fragment (black line) and with control – PCR amplified 16S rRNA gene fragment (dashed line)

SWV measurements carried out for the samples with PCR amplified aerA gene fragment and with control – PCR amplified 16S rRNA gene fragment allow the successful discrimination between both examined solutions. It was presented on the figure 4 the $Co(bpy)_3^{3+}$ signals difference between these two experiments was 0.2 μA. This evidence that elaborated biosensor is the useful device for the detection of nucleotide sequence specific to aerA gene fragment.

Conclusions

Identification of *Aeromonas hydrophila* is difficult, requires a series of biochemical tests implementation, because this pathogen presents broad metabolic capabilities. To overcome these difficulties we developed a some molecular methods as PCR, Southern blotting and electrochemical DNA biosensor for the detection of aerolysin gene, toxin produced by *A. hydrophila*. These methods would help to ensure rapid and accurate detection of pathogenic strains of *A. hydrophila*.

Acknowledgements

This work was financially supported by the Polish Ministry of Science and High Education. Grant No. N N312 206736.

References

Arnheim N., Erlich H., 1992. Polymerase chain reaction strategy. Annual Rev. Biochem, 61, 131–156.

Asao T., Kinoshita Y., Kozaki S., Uemura T., Sakaguchi G., 1984. Purification and some properties of *Aeromonas hydrophila* hemolysin. Infect. Immun., 46, 122–127.

Bernheimer A.W., Avigad L.S., 1974. Partial characterization of aerolysin, a lytic exotoxin from *Aeromonas hydrophila*. Infect. Immun., 9, 1016–1021.

Burke V., Robinson J., Berry R.J., Gracey M., 1981. Detection of enterotoxins of *Aeromonas hydrophila* by a suckling-mouse test. J. Med. Microbiol., 14, 401–408.

Chakraborty T., Huhle B., Bergbauer H., Goebel W., 1986. Cloning, expression, and mapping of the *Aeromonas hydrophila* aerolysin gene determinant in *Escherichia coli* K-12. J. Bacteriol., 167, 368–374.

de-los-Santos-Álvarez P., Lobo-Castañón M.J., Miranda-Ordieres A.J., Tuñón-Blanco P., 2004. Current strategies for electrochemical detection of DNA with solid electrodes. Anal. Bioanal. Chem., 378, 104–118.

Di Bari M., Hachich Elayse M., Melo Adalgisa M.J., Sato M.I.Z., 2007. *Aeromonas spp*. and microbial indicators in raw drinking water source. Brazilian J. Microbiol., 38, 516–521.

Ferguson M.R., Xu X.J., Houston C.W., Peterson J.W., Coppenhaver D.H., Popov D.V., Chopra A.K., 1997. Hyperproduction, purification, and mechanism of action of the cytotoxic enterotoxin produced by *Aeromonas hydrophila*. Infect. Immun., 65, 4299–4308.

Figueras M.J., 2005. Clinical relevance of *Aeromonas*. Rev. Med. Microbiol., 16,144–153.

Galindo C.L., Sha J., Fadl A.A., Pillai L., Chopra A.K., 2006. Host immune responses to *Aeromonas* virulence factors. Curr. Immunol. Rev., 2, 13–26.

Galindo C.L., Chopra A.K., 2007. *Aeromonas* and *Plesiomonas* species, in: *Food Microbiology fundamentals and frontiers* (Doyle M.P. and Beuchat L.R., Eds.). ASM Press. Washington, 381–401.

González-Serrano C.J., Santos J.A., Garcīa-Lõpez M.A., Otero A., 2002. Virulence markers in *Aeromonas hydrophila* and *Aeromonas veronii biovar sobria* isolates from freshwater fish and from a diarrhoea case. J. Appl. Microbiol., 93, 414–419.

Hall R.H., 2002. Biosensor technologies for detecting microbiological foodborne hazards. Microbes Infec., 4, 425–432.

Hiransuthikul N., Tantisiriwat W., Lertutsahakul K., Vibhagool A., Boonma P., 2005. Skin and soft-tissue infections among tsunami survivors in southern Thailand. Clin. Infect. Dis., 41, 93–96.

Howard S.P., Garland W.J., Green M.J., Buckley J.T., 1987. Nucleotide sequence of the gene for the hole-forming toxin aerolysin of *Aeromonas hydrophila*. J. Bacteriol., 169, 2869–2871.
Janda J.M., Abbott S.L., 1998. Evolving concepts regarding the genus *Aeromonas*: an expanding panorama of species, disease presentations, and unanswered questions. Clin. Infect. Dis., 27, 332–344.
Johnson W.M., Lior H., 1981. Cytotoxicity and suckling mouse reactivity of *Aeromonas hydrophila* isolated from human sources. Can. J. Microbiol., 27, 1019–1027.
Ligaj M., Jasnowska J., Musiał W.G., Filipiak M., 2006. Covalent attachment of DNA fragments to carbon paste electrode modified by activated carboxyl groups. Electrochim. Acta, 51, 5193–5198.
Ljungh A., Wadstrom T., 1983. Toxins of *Vibrio parahaemolyticus* and *Aeromonas hydrophila*. J. Toxicol. Toxin Rev., 1, 257–307.
Martin-Carnahan A., Joseph S.W., 2005. *Aeromonadaceae*, [in:] Bergeys Manual of Systematic Bacteriology (Brenner D.J., Krieg N.R., Staley J.T., Garrity G.M., Eds.). Springer. New York, 556–578.
Morgan D.R., Johnson P.C., Du Pont H.L., Satterwhite T.K., Wood L.V., 1985. Lack of correlation between known virulence properties of *Aeromonas hydrophila* and enteropathogenicity for humans. Infect. Immun., 50, 62–65.
Naharro G., Riano J., de Castro L., Alvarez S., Luengo J.M., 2009. *Aeromonas*, [in:] Molecular Detection of Foodborne Pathogens (Liu D. Ed.). CRC Press. North Ryde, 273–289.
Napierała D., Kaczmarek M., Jura J., Słomski R., 2004. PCR multipleks, [in:] Przykłady analiz DNA (Słomski R. Ed). University of Life Sciences Press. Poznań, 139–144.
Palumbo S.A., Bencivengo M.M., Del Corral F., Williams A.C., Buchanan R.L., 1989. Characterization of the *Aeromonas hydrophila* group isolated from retail foods of animal origin. J. Clin. Microbiol., 27, 854–859.
Pividori M.I., Merkoçi A., Alegret S., 2000. Electrochemical genosensor design: immobilisation of oligonucleotides onto transducer surfaces and detection methods. Biosens. Bioelectron., 15, 291–303.
Pollard D.R., Johnson W.M., Lior H., Tyler S.D., Rozee K.R., 1990. Detection of the Aerolysin Gene in *Aeromonas hydrophila* by the Polymerase Chain Reaction. J. Clinical Microbiol., 28, 2477–2481.
Presley S.M., Rainwater T.R., Austin G.P., Platt S.G., Zak J.C., Cobb G.P., Marsland E.J., Tian K., Zhang B., Anderson T.A., Cox S.B., Abel M.T., Leftwich B.D., Huddleston J.R., Jeter R.M., Kendall R.J., 2006. Assessment of pathogens and toxicants in New Orleans, LA following Hurricane Katrina. Environ. Sci. Technol., 40, 468–474.
Rose J.M., Houston C.W., Coppenhaver D.H., Dixon J.D., Kurosky A., 1989. Purification and chemical characterization of a cholera toxin-cross-reactive cytolytic enterotoxin produced by a human isolate of *Aeromonas hydrophila*. Infect. Immun., 57, 1165–1169.
Sen K., Rodgers M., 2004. Distribution of six virulence factors in *Aeromonas* species isolated from US drinking water utilities: a PCR identification. J. Appl. Microbiol., 97, 1077–1086.
Seshadri R., Joseph S.W., Chopra A.K., Sha J., Shaw J., Graf J., Haft D., Wu M., Ren Q., Rosovitz M.J., Madupu R., Tallon L., Kim M., Jin S., Vuong H., Stine O.C., Ali A., Horneman A.J., Heidelberg J.F., 2006. Genome sequence of *Aeromonas hydrophila* ATCC 7966 T: Jack of All Trades. J. Bacteriol., 12, 8272–8282.
Singh V., Somvanshi P., Rathore G., Kapoor D., Mishra B.N., 2009. Gene cloning, expression and homology modeling of hemolysin gene from *Aeromonas hydrophila*. Protein Express. Purific., 65, 1–7.
Vally H., Whittle A., Cameron S., Dowse G. K., Watson T., 2004. Outbreak of *Aeromonas hydrophila* wound infections associated with mud football. Clin. Infect. Dis., 38, 1084–1089.
Wang G., Clark C.G., Liu C., Pucknell C., Munro C.K., Kruk T.M., Caldeira R., Woodward D.L., Rodgers F.G., 2003. Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR, J. Clin. Microbiol., 3, 1048–1054.
Wang J., 1999. Towards Genoelectronics: Electrochemical Biosensing of DNA Hybridization. Chem. Eur. J. 5, 1681–1685.
Yogananth N., Bhakyaraj R., Chantchuru A., Anbalagan T., Mullai Nila K., 2009. Detection of virulence gene in *Aeromonas hydrophila* isolated from fish samples using PCR technique. Global J. Biotechnol. Biochem., 4, 51–53.

2

USING OF MULTIPLEX PCR METHOD FOR DETECTION OF *CLOSTRIDIUM BOTULINUM* IN FOOD AND FEED

Introduction

The bacteria from the species *Clostridium botulinum* are spore – forming rods, which have the ability to produce the most potent toxic substances in environment, which are named botulinum toxins. These toxins are etiological factor of botulism. The most frequently symptoms of this disease are connected with consumption by human or animal food or feed contaminated by spores or toxins produced by *C. botulinum.* In the most cases the methods for detection of this microorganism are based on proving the ability of isolated strains to produce botulinum toxins. The isolation of *C. botulinum* is complicated because of occurrence of the other microorganisms which are phenotypically similar to this species, however are not able to produce botulinum toxins. Until now, there are no any selective media for isolation of *C. botulinum* [Anon. 2002]. Clostridium botulinum. International Programme on Chemical Safety Poisons Information. Monograph 858. Bacteria. World Health Organization (WHO) 2002, Hatheway 1995, Hatheway 1990, Saeed 2004, Vu 2006).

The methods based on PCR techniques enable detection of genes which determine the production of botulinum toxins. These methods characterize high sensitivity and specificity [Lindström and Korkeala 2006, Saeed 2004, Vu 2006].

The detection of *C. botulinum* is possible at the different stages of culturing process or directly from sample (without enrichment, that is *in situ*). The most PCR protocols are based on detection of *bont* genes, which determine the production of active form of botulinum toxins (BoNT) – specific for particular toxinotypes of this pathogen. There are also known protocols based on detection of *ntnh* gene, which determine production of non toxic non haemagglutinin component in botulinum protoxin. This component is common in all toxinotypes of *C. botulinum* [Lindström and Korkeala 2006].

The most of PCR protocols are based on detection of single *bont* genes for particular toxinotypes. In the literature, there are also described numerous protocols of multiplex PCR methods, which enable the detection of several toxinotypes in one probe [Lindström et al. 2001, Lindström and Korkeala 2006].

The aim of this study was *in house* validation of mPCR method for detection of *Clostridium botulinum* in feed and food matrixes.

Material and methods

This study was carried out on food (fish salad) and feed matrixes (ground grain) which were contaminated by spores of reference *C. botulinum* strains, that were: *C. botulinum* NCTC 887 (type A), *C. botulinum* NCTC 3815 (type B), *C. botulinum* NCTC 8266 (type E), *C. botulinum* NCTC 10281 (type F). The spores of mentioned strains were cropped according methods described by Fletcher et al. 2008.

The results were estimated according to the PN – EN – ISO – 16140 [Anon. 2004. Polish Standard: Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods. PN-EN ISO 16140]. The 10 g of each contaminated sample was incubated in 90 ml of TPGY broth during 48 h at 37^{o}C, in the anaerobic conditions. Extraction of DNA was performed from 1ml of culture by using the commercial kit Genomic Mini AX Bacteria (A&A Biotechnology). The obtained DNA was submitted to the examination by using mPCR method.

The mentioned mPCR method enabled detection of *bont* genes of A, B, E, and F toxinotypes of *C. botulinum,* the set of eight primers was used (Table 1), according to the CEN [Anon. 2008: Draft. Polymerase Chain Reaction (PCR) for the detection of botulinum eurotoxin – producing clostridia w CEN/TC 275/WG 6 DOC N368.15th meeting of CEN/TC 275/WG 6, Helsinki – Finland]. This set allowed the detection of a gene, which creates active component of botulinum toxin production (BoNT) in particular toxinotypes. The sequences of primers are summarized in Table 1. The final volume of reaction mixture was 25 µl and contained: 5 µl of DNA template, 2.5 µl of 10xTaq buffer with KCl (Fermentas), 0.3 µM of each primer, 4 mM of MgCl2 (Fermentas), 200 µM of dNTP mixture (Fermentas), and 1.25U

Table 1

Primers for detection of *Clostridium botulinum* by using multiplex PCR

Toxinotype	Primer	Sequence	Lenght of PCR product	Literature
A	IA_03_fw	5'GGGCCTAGAGGTAGCGTARTG'3	101 bp	11
	IA_03_rev	5''TCTTYATTTCCAGAAGCATATTTT' 3		
B	CBMLB1	5'CAGGAGAAGTGGAGCGAAAA'3	205 bp	16
	CBMLB2	5'CTTGCGCCTTTGTTT TCT TG '3		
E	CBMLE1	5'CCAAGATTTTCATCCGCCTA'3	389 bp	
	CBMLE2	5'GCTATTGATCCAAAACGGTGA'3		
F	CBMLF1	5'CGGCTTCATTAGAGAACGGA'3	543 bp	
	CBMLF2	5'TAACTCCCCTAGCCCCGTAT '3		

of Taq DNA polymerase (Fermentas). Thermocycling was performed on the T1 thermocycler instrument (Biometria). Following initial denaturation step at 95°C for 60 s, reaction was subjected to 27 cycles at 95°C for 30 s, at 53°C for 30 s, and at 72°C for 3 min. Final extension was carried out at 72°C for 3 min. Detection of mPCR products was performed on agarose gel (Fig.1).

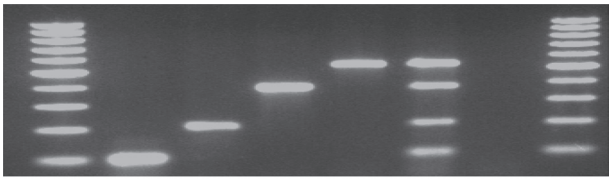


Fig. 1. Characteristic products of mPCR for the A, B, E and F toxinotypes
Where:
M – Molecular mass standard
1 – *Clostridium botulinum,* toxinotype A (101 bp); 2 – *Clostridium botulinum,* toxinotype B (205 bp);
3 – *Clostridium botulinum,* toxinotype E (389 bp); 4 – *Clostridium botulinum,* toxinotype F (543 bp);
5 – *Clostridium botulinum* A, B, E, F; C- – negative PCR control

The mPCR products were separated in 2% agarose gel stained with ethidium bromide, located in 1xTBE buffer (Fermentas) for 1.5 h under 100 V. Into each well, 10 µl of reaction mixture and 2 µl of loading buffer 6xDNA Loading Dye (Fermentas) were loaded. The molecular weight of the obtained products was determined on the basis of a molecular weight marker, which was GeneRuler™ 100bp DNA Ladder Mix (Fermentas). After separation, PCR products were analysed under an UV light transluminator (Vilber-Lourmat).

The values of limit of detection and sensitivity were estimated for three contamination levels (Tab. 2). At each level 20 samples of contaminated feed and food matrixes were analyzed.

The specificity of method was estimated by examination of 20 samples of non – contaminated feed and food matrixes by *C. botulinum* spores, and by examination of DNA obtained from the other strains of *Clostridia*, that were: *C. chavouei, C. tetani, C. septicum, C. sporogenes, C. oedematiens, C. sordelli, C. pasteurianum, C. novyi, C. fallax, C. histolyticum* (own isolates) and *C. perfringens* (ATCC 13124).

The accuracy was estimated for mentioned three levels of contamination (Tab. 2) and non – contaminated samples.

Table 2

Contamination levels

Kind of sample		Fish salad samples				Ground grain samples			
Toxinotype		A	B	E	F	A	B	E	F
Contamination levels Spore(cfu)/g	Level I	0.3	0.5	1	0,7	3	5	10	7
	Level II	0.03	0.05	0.1	0.07	0.3	0.5	1	0.7
	Level III	0.003	0.005	0.01	0.007	0.03	0.05	0.1	0.07

The limit of detection expressed as LOD_{50} for particular toxinotypes in feed and food matrixes, was statistically estimated with using Spaerman – Kärber method, that is number of microorganisms per gram of sample at which 50% of the tests are positive and 50% are negative.

During the examinations guidelines from AOAC documents were used [Anon. 2006, Final report and executive summaries from the AOAC international: Presidential Task Force on Best Practices in Microbiological Methodology. AOAC international. Gaithersburg], and from the other publications [Anon. 2009, Protocol for the validation of alternative microbiological methods. NordVal. Norway; Carter 1994, Hamilton et al. 1977]. The estimation of LOD_{50} was conducted with using excel calculator of LOD_{50} available at www.aoac.org/accreditation/DEMO.xls. The obtained results were expressed in the 95% confidential interval.

For determination of LOD_{50} there were used bellowed formulas:

- Estimation of LOD_{50}

$$\tilde{\mu} = \sum_{i=1}^{k-1} (p_{i+1} - p_i)(x_i + x_{i+1})/2$$

$$\log(LOD_{50}) = \tilde{\mu}$$

$$LOD_{50} = 10^{\tilde{\mu}}$$

- Estimation of 95% confidential interval for suspected value of LOD_{50}

$$\mathrm{var}(\tilde{\mu}) = \sum_{i=2}^{k-1} [p_i q_i / (n_i - 1)] \; [(x_{i+1} - x_{i-1})/2]^2$$

$$\sqrt{\mathrm{var}(\tilde{\mu})} = s$$

$$\ln(LOD_{50}) \in (\ln(LOD_{50}) - 2{,}303(1{,}96s); \ln(LOD_{50}) + 2{,}303(1{,}96s))$$

Where:

$\tilde{\mu}$ – LOD_{50} estimator;

$\mathrm{var}(\tilde{\mu})$ – variation of LOD_{50} estimator;

k – number of contamination levels;

p_i – sensitivity for each analyzed level (i = 1...k);

x_i – log microorganism number at the given level ($x_i < \ldots < x_k$);

$q_i = 1 - p_i$;

n_i – number of examined samples at given level;

s – standard deviation;

1,96 – value read out from t – student tables for level of relevance $\alpha = 0{,}05$ and undetermined number of freedom degree at 95% confidential interval;

2,303 – approximate value of conversion rate of decimal into natural logarithm.

Specificity (SP) was estimated as the rate of negative samples, evaluated in the examination as negative (NA), to the total number of negative results (N _)

$$SP = \frac{NA}{N\_} \times 100\%$$

Sensitivity (SE) was estimated as the rate of positive samples, evaluated in the examination as positive (PA), to the total number of positive results (N+).

$$SE = \frac{PA}{N+} \times 100\%$$

Accuracy (AC) was estimated as the rate of sum of positive samples, evaluated in the examination as positive (PA) and negative samples evaluated in the examination as negative (NA), to the total number of samples (N).

$$AC = \frac{(PA + NA)}{N} \times 100\%$$

Results

The examination of fish salad samples contaminated by spores of reference strain *C. botulinum* NCTC 887 shown that the obtaining 100% sensitivity was possible for level 0.3 spore/g. The sensitivity at the lower levels of contamination were estimated as 65% for 0.03 spore/g and 10% for 0.003 spore/g. The limit of detection expressed as LOD_{50} was estimated at the level of 0.034 (0.021 – 0.056) spore/g. The accuracy for the detection of *C. botulinum* NCTC 887 in the fish salad samples was estimated at the level 69% (Tab. 3). The pictures of obtained products at mentioned levels of contamination shown Figure 2.

Table 3

Values of characteristic parameters for detection of *C. botulinum* NCTC 887 (toxinotype A) in fish salad samples

Level of contamination (spore/g)	N	N+	N_	PA	NA	$SP = \frac{NA}{N\_} \times 100\%$	$SE = \frac{PA}{N+} \times 100\%$	$CA = \frac{(PA + NA)}{N} \times 100\%$	LOD_{50} (spore/g)
0.3	20	20	–	20	–	–	100%	69%	0.034 (0.021 – 0.056)
0.03	20	20	–	13	–	–	65%		
0.003	20	20	–	2	–	–	10%		
0	20	–	20	–	20	100%	–		

The example of estimation of LOD_{50} in accordance with the calculation obtaining by using AOAC calculator available on – line (www.aoac.org/accreditation/DEMO.xls):

- Estimation of LOD_{50} :

$$\tilde{\mu} = \sum_{i=1}^{k-1} (p_{i+1} - p_i)(x_i + x_{i+1})/2$$

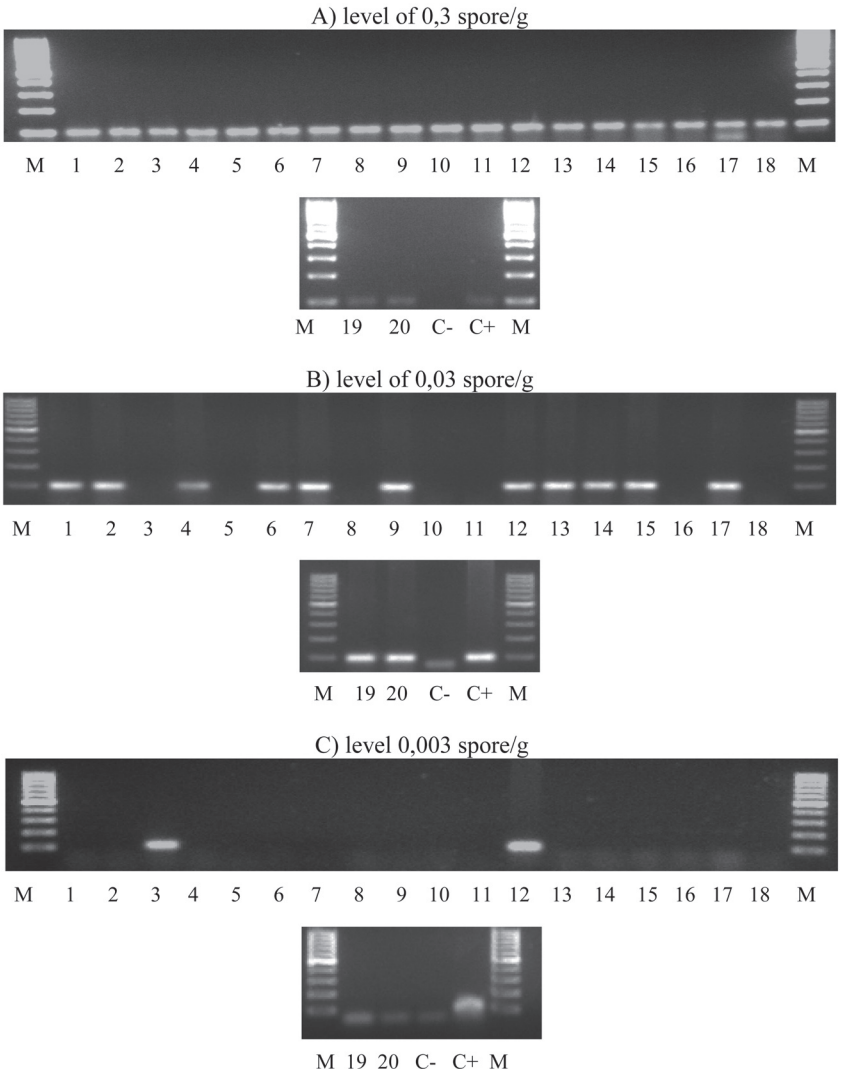


Fig. 2. Pictures shown sensitivity of mPCR in the detection of *Clostridium botulinum* NCTC 887 from fish salad samples at the three levels of contamination (A, B, C)
Where:
M – molecular mass standards,
1 – 20 – the results obtained for fish salad samples contaminated by spores of *Clostridium botulinum* NCTC 887 (101 pz),
C+ – positive PCR control,
C- – negative PCR control

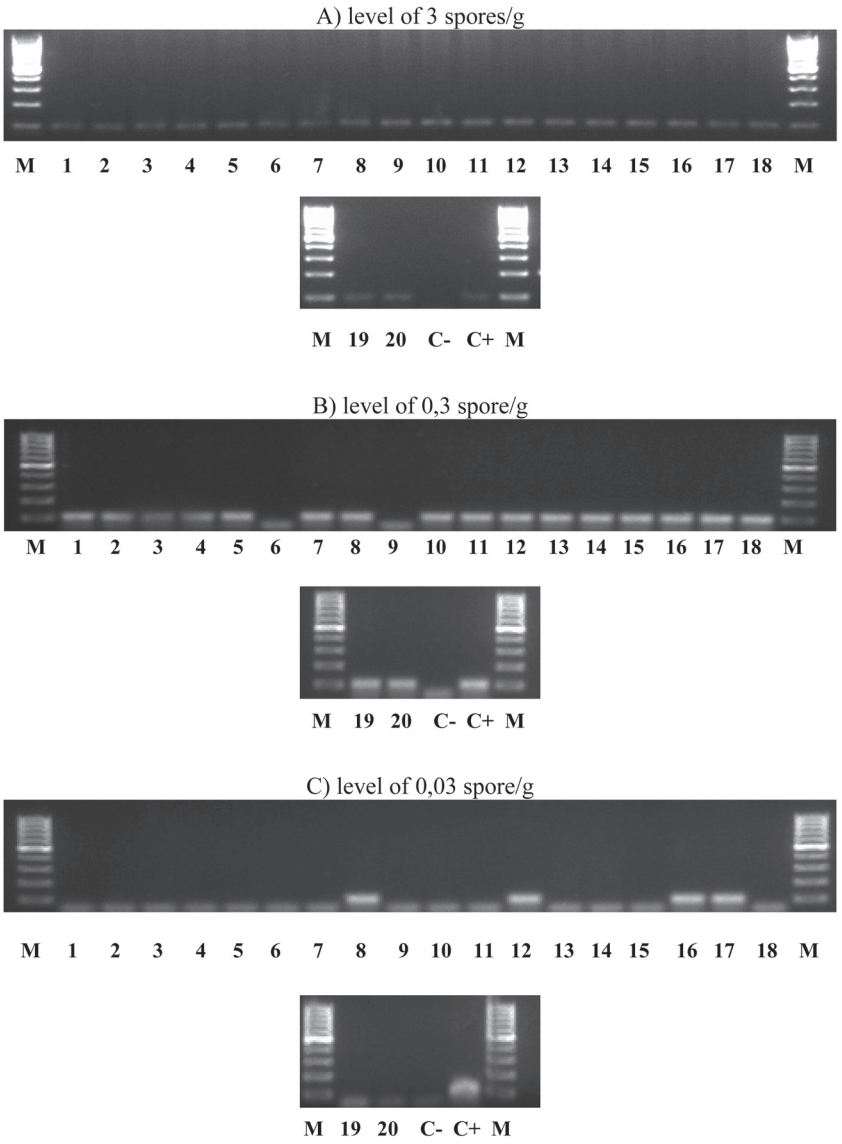


Fig. 3. Pictures shown sensitivity of mPCR in the detection of *Clostridium botulinum* NCTC 887 from ground grain samples at the three levels of contamination (A, B, C)
Where:
M – molecular mass standards,
1 – 20 – the results obtained for grain slurry samples contaminated by spores of *Clostridium botulinum* NCTC 887 (101 pz),
C+ – positive PCR control,
C- – negative PCR control.

- $\tilde{\mu}_i$ for i = 0.003 spor/g:

$p_{0.003}$	$p_{0.03}$	$x_{0.003}$	$x_{0.03}$
2/20 = 0.1	13/20 = 0.65	log (0.003) = -2.523	log (0.03) = -1.523

$$\tilde{\mu}_{0,003} = (p_{0.0} - p_{0.003})(x_{0.003} + x_{0.0})/2$$

$$\tilde{\mu}_{0,003} = (p_{0.03} - p_{0.003})(x_{0.003} + x_{0.03})/2$$

$$\tilde{\mu}_{0,003} = (0.65 - 0.1)(-2.523 - 1.523)/2$$

$$\tilde{\mu}_{0,003} = -1.112$$

- $\tilde{\mu}_i$ for i = 0.03 spore/g:

$p_{0.03}$	$p_{0.3}$	$x_{0.03}$	$x_{0.3}$
13/20 = 0.65	20/20 = 1	log(0.03) = -1.523	log(0.3) = -0.523

$$\tilde{\mu}_{0.03} = (p_{0.3} - p_{0.03})(x_{0.03} + x_{0.3})/2$$

$$\tilde{\mu}_{0.03} = (1 - 0.65)(-1.523 - 0.523)/2$$

$$\tilde{\mu}_{0.03} = (0.35)(-2.046)/2$$

$$\tilde{\mu}_{0,03} = -0,358$$

- $\tilde{\mu} = \tilde{\mu}_{0.003} + \tilde{\mu}_{0.03}$

$$\tilde{\mu} = -1.112 - 0.358$$

$$\tilde{\mu} = -1.47$$

$$\log(LOD_{50}) = -1.47$$

$$LOD_{50} = 10^{-1,47}$$

$$LOD_{50} = 0.034 \text{ spore/g}$$

- Estimation of 95% confidential interval for the obtained value of LOD_{50}:

$$\ln(LOD_{50}) = 2.303(-1.47)$$

$$\ln(LOD_{50}) = -3.385$$

$$\text{var}(\tilde{\mu}) = \sum_{i=2}^{k-1} [p_i q_i / (n_i - 1)] \; [(x_{i+1} - x_{i-1})/2]^2$$

$$\sqrt{\text{var}(\tilde{\mu})} = s$$

- $\text{var}(\tilde{\mu})$ for i = 0,03 spore/g:

$p_{0.03}$	$n_{0.03}$	$q_{0.03}$	$x_{0.3}$	$x_{0.003}$
13/20=0.65	20	1-0.65=0.35	log(0.3) = -0.523	log(0.003) = -2.523

$$\text{var}(\tilde{\mu})_{0.03} = [p_{0.03} q_{0.03} / (n_{0.03} - 1)] \; [(x_{0.3} - x_{0.003})/2]^2$$

$$\text{var}(\tilde{\mu})_{0.03} = [0.65x0.35/(20-1)]\ [(-0.523+2.523)/2]^2$$

$$\text{var}(\tilde{\mu})_{0.03} = 0.012$$

$$s = \sqrt{\text{var}(\tilde{\mu})_{0.03}} = 0.1$$

➢ $$\ln(LOD_{50}) \in \left(\ln(LOD_{50}) - 2.303(1.96s); \ln(LOD_{50}) + 2.303(1.96s) \right)$$

$$\ln(LOD_{50}) \in \left(-3.385 - 2.303(1.96(0.1)); \ -3.385 + 2.303(1.96(0.1)) \right)$$

$$\ln(LOD_{50}) \in \left(-3.881; \ -2.888 \right)$$

$$LOD_{50} \in \left(e^{-3.881}; e^{-2.888} \right) \text{ spore/g}$$

$$LOD_{50} \in \left(0.021; \ 0.056 \right) \text{ spore/g}$$

0.034 (0.021 – 0.056) spore/g

During analysis of fish salad samples, contaminated by spores of *C. botulinum* NCTC 3815, obtaining of 100% sensitivity was possible at the level of 0.5 spore/g. At the lower levels of contamination 0.05 spore/g and 0.005 spore/g, this parameter reached the adequately values of 75 and 5%. The limit of detection expressed as LOD_{50} was estimated at the level of 0.035 (0.022–0.054) spore/g. The accuracy for the detection of *C. botulinum* NCTC 3815 in the fish salad samples obtained 70% (Table 4).

Table 4

Values of characteristic parameters for detection of *C. botulinum* NCTC 3815 (toxinotype B) in fish salad samples

Level of contamination (spore/g)	N	N+	N_	PA	NA	$SP = \frac{NA}{N\_} \times 100\%$	$SE = \frac{PA}{N+} \times 100\%$	$CA = \frac{(PA+NA)}{N} \times 100\%$	LOD_{50} (spore/g)
0.5	20	20	–	20	–	–	100%	70%	0.035 (0.022 – 0.054)
0.05	20	20	–	15	–	–	75%		
0.005	20	20	–	1	–	–	5%		
0	20	–	20	–	20	100%	–		

For the contaminated fish salad samples, by *C. botulinum* NCTC 8266 spores, sensitivity obtained 100% at the level of 1 spore/g. At the lower levels – 0.1 spore/g and 0.01 spore/g this parameter reached the adequately values of 90% and 30%. The limit of detection expressed as LOD_{50} was estimated at the level 0.094 (0.069 – 0.129)spore/g. The accuracy for the detection of *C. botulinum* NCTC 8266 in the fish salad samples was estimated at the level of 79% (Tab. 5).

The examination of food matrix samples contaminated by spores of *C. botulinum* NCTC 10281 shown that the obtaining of 100% sensitivity was possible at the level of

Table 5

Values of characteristic parameters for detection of *C. botulinum* NCTC 8266 (toxinotype E) in fish salad samples

Level of contamination (spore/g)	N	N+	N_	PA	NA	$SP = \frac{NA}{N\_} \times 100\%$	$SE = \frac{PA}{N+} \times 100\%$	$CA = \frac{(PA + NA)}{N} \times 100\%$	LOD_{50} (spore/g)
1	20	20	–	20	–	–	100%	79%	0.094 (0.069 – 0.129)
0.1	20	20	–	18	–	–	90%		
0.01	20	20	–	5	–	–	20%		
0	20	–	20	–	20	100%	–		

0.7 spore/g. For the level 0.07 spore/g sensitivity obtained value 50%, whilst for 0.007 spore-/g – 10%. The limit of detection expressed as LOD_{50} was estimated at the level of 0.102(0.062 – 0.168) spore/g. The accuracy for the detection of *C. botulinum* NCTC 10281 in the fish salad samples obtained level of 65% (Tab. 6).

Table 6

Values of characteristic parameters for detection of *C. botulinum* NCTC 10281 (toxinotype F) in fish salad samples

Level of contamination (spore/g)	N	N+	N_	PA	NA	$SP = \frac{NA}{N\_} \times 100\%$	$SE = \frac{PA}{N+} \times 100\%$	$CA = \frac{(PA + NA)}{N} \times 100\%$	LOD_{50} (spore/g)
0.7	20	20	–	20	–	–	100%	65%	0.102 (0.062 – 0.168)
0.07	20	20	–	10	–	–	50%		
0.007	20	20	–	2	–	–	10%		
0	20	–	20	–	20	100%	–		

During analysis of the ground grain, contaminated by *C. botulinum* NCTC 887 spores, reaching of 100% sensitivity was possible at the level of 3 spores/g. At the level of 0.3 sensitivity obtained 90% and at 0.03 spore/g – 20%. The limit of detection expressed as LOD_{50} was estimated at the level of 0.191 (0.139–0.262) spore/g. The accuracy for detection of *C. botulinum* NCTC 887 in the ground grain samples reached the level of 77% (Tab. 7).

Table 7

Values of characteristic parameters for detection of *C. botulinum* NCTC 887 (toxinotype A) in ground grain samples

Level of contamination (spore/g)	N	N+	N_	PA	NA	$SP = \frac{NA}{N\_} \times 100\%$	$SE = \frac{PA}{N+} \times 100\%$	$CA = \frac{(PA + NA)}{N} \times 100\%$	LOD_{50} (spore/g)
3	20	20	–	20	–	–	100%	77%	0.191 (0.139 – 0.262)
0.3	20	20	–	18	–	–	90%		
0.03	20	20	–	4	–	–	20%		
0	20	–	20	–	20	100%	–		

For the contaminated ground grain samples, by spores of *C. botulinum* NCTC 3815, reaching of 100% sensitivity was possible at the level of 5 spores/g. For 0.5 spore/g the sensitivity reached 80% and for 0.05 spore/g – 15% The limit of detection expressed as LOD_{50} was estimated at the level of 0.251 (0.167–0.376) spore/g. The accuracy for the detection of *C. botulinum* NCTC 3815 in ground grain samples reached 65% (Tab. 8).

Table 8

Values of characteristic parameters for detection of *C. botulinum* NCTC 3815 (toxinotype B) in ground grain samples

Level of contamination (spore/g)	N	N+	N_	PA	NA	$SP = \frac{NA}{N\_} \times 100\%$	$SE = \frac{PA}{N+} \times 100\%$	$CA = \frac{(PA+NA)}{N} \times 100\%$	LOD_{50} (spore/g)
5	20	20	–	20	–	–	100%	65%	0.251 (0.167 – 0.376)
0.5	20	20	–	16	–	–	80%		
0.05	20	20	–	3	–	–	15%		
0	20	–	20	–	20	100%	–		

The examination of feed matrix samples contaminated by *C. botulinum* NCTC 8266 shown, that obtaining 100% of sensitivity was possible at the level of 10 spores/g. At the level of 1 spore/g sensitivity obtained 55% and for 0.1 spore/g – 0%. The limit of detection expressed as LOD_{50} was estimated at the level of 0.891 (0.592–1.464) spore/g. The accuracy for the detection of *C. botulinum* NCTC 8266 in the feed matrix reached 64% (Tab. 9).

Table 9

Values of characteristic parameters for detection of *C. botulinum* NCTC 8266 (toxinotype E) in ground grain samples

Level of contamination (spore/g)	N	N+	N_	PA	NA	$SP = \frac{NA}{N\_} \times 100\%$	$SE = \frac{PA}{N+} \times 100\%$	$CA = \frac{(PA+NA)}{N} \times 100\%$	LOD_{50} (spore/g)
10	20	20	–	20	–	–	100%	64%	0.891 (0.542 – 1.464)
1	20	20	–	11	–	–	55%		
0.1	20	20	–	0	–	–	0%		
0	20	–	20	–	20	100%	–		

The sensitivity for the samples of ground grain contaminated by *C. botulinum* NCTC 10281 was estimated as 100% at the level of 7 spores/g. At the level of 0.7 spore/g this parameter reached the level 85% and at 0.07 spore/g – 20%. The limit of detection expressed as LOD_{50} was estimated at the level 0.423 (0.295–0.607) spore/g. The accuracy for the detection of *C. botulinum* NCTC 10281 in the ground grain reached 76% (Tab. 10).

The specificity estimated for non – contaminated fish salad and ground grain samples reached 100% (Tab. 2 – 10). Specific products of mPCR were only obtained for DNA extracted from samples contaminated by *C. botulinum* spores. There were no any positive results for DNA obtained from the other strains of *Clostridia.*

Table 10

Values of characteristic parameters for detection of *C. botulinum* NCTC 10281 (toxinotype F) in ground grain samples

Level of contamination (spore/g)	N	N+	N_	PA	NA	$SP = \frac{NA}{N\_} \times 100\%$	$SE = \frac{PA}{N+} \times 100\%$	$CA = \frac{(PA + NA)}{N} \times 100\%$	LOD_{50} (spore/g)
7	20	20	–	20	–	–	100%	76%	0.423 (0.295 – 0.607)
0.7	20	20	–	17	–	–	85%		
0.07	20	20	–	4	–	–	20%		
0	20	–	20	–	20	100%	–		

Discussion

The results shown differences in the limits of detection (LOD_{50}) of *C. botulinum* spores in the food and feed matrixes. During examination of contaminated fish salad samples – the lowest values of this parameter were estimated for A and B and the highest for E and F toxinotypes.

The results obtained from the analyses of ground grain samples contaminated with *C. botulinum* spores shown significant inhibition of PCR and the growth of *C. botulinum* strains which had influence to the higher limits of detection. In comparison to the results obtained for the contaminated samples of fish salad, values of LOD_{50} were approx. 10 times higher. The lowest values of LOD_{50} were estimated for A and B toxinotypes and the highest for E and F.

The highest values of LOD_{50} obtained from analysis of contaminated ground grain samples could be linked with effect of matrix ingredients, which have inhibitory influence to the growth of *C. botulinum* and mPCR. The relatively high values of LOD_{50}, obtained for detection of *C. botulinum* toxinotype E, could be related to the influence of temperature used in the experiments. For the incubation of samples – temperature of 37°C was chosen, which is optimal for the growth of proteolytic strains of *C. botulinum*. In the most cases, proteolytic A and B strains are connected with botulism in human and animals (except C and D toxinotypes). These strains produce proteases which relieve active components of botulinum toxins (BoNT) from the protoxin complex and do not need action of proteolytic enzymes produced by host organism (animal or human). The optimal temperatures for toxinotype E ranged from 18–25°C [Hathawey 1995, Hathaway 1990, Saeed 2004]. The problems connected with inhibitory effect of matrix ingredients during detection *C. botulinum* were also reported by the other authors, which described detection of this pathogen after liquid culturing. Lindström et al. [2001] described multiplex PCR method for detection of A, B, E, F toxinotypes in food and faeces. The limit of detection for faeces samples obtained levels from 10^{-1} to 10^{3} spore/g, whilst for fish and meat samples ranged from 10^{-2} to 10^{-1} spore/g. Fach et al. [2002] reported PCR method for detection of A, B, E, F in food samples with the limit of detection 10^{-1} cfu/g. Dahlenborg et al. [2003] shown difficulties with detection of *C. botulinum* spores from faecal samples, which was possible at the high levels of $10^{1} – 10^{2}$ spore/g.

The validated methods enabled specific analyses for detection of *C. botulinum.* The specific products were only obtained for reference strains of this pathogen.

The described mPCR method, despite limitation connected with inhibitory influence of matrixes to the obtained results, improve the detection of *C. botulinum* in food and feed. The examination of samples could be conducted without the isolation process. Nowadays in Poland, there is lack of proper methods for the detection of this pathogen. The most frequently used is ethically controversial and time – consuming test on laboratory mice [Anon. 1994, Polish Standard: Animal feeding stuffs – Requirements and microbiological examinations. PN – R – 64791; Anon. 1997, Polish Standard: Meat and meat products – Microbiological examinations – Detection of anaerobic spore forming bacteria and anaerobic spore forming bacteria of sulphite reducing. PN – A – 82055 – 12]. The analyses by using described method make possible limiting time of detection to 3 days. The described mPCR test could be used for supporting diagnostic of botulism in human and animals. However, this method is only tool for detection of *C. botulinum*. For the providing food and feed safety and the effective laboratory diagnostic of botulism, there is also needed development of methods for detection of botulinum toxins.

Acknowledgements

This work was financially supported by the Polish Ministry of Science and High Education. Project No N N308 563639 (2010 – 2012).

References

Anon., 2002. *Clostridium botulinum.* International Programme on Chemical Safety Poisons Information. Monograph 858. Bacteria. World Health Organization (WHO).

Anon., 2008. Draft. Polymerase Chain Reaction (PCR) for the detection of botulinum eurotoxin – producing clostridia w CEN/TC 275/WG 6 DOC N368.15th meeting of CEN/TC 275/WG 6, Helsinki – Finland.

Anon., 2006. Final report and executive summaries from the AOAC international: Presidential Task Force on Best Practices in Microbiological Methodology. AOAC international. Gaithersburg.

Anon., 1994. Polish Standard: Animal feeding stuffs – Requirements and microbiological examinations. PN – R – 64791.

Anon., 1997. Polish Standard: Meat and meat products – Microbiological examinations – Detection of anaerobic spore forming bacteria and anaerobic spore forming bacteria of sulphite reducing. PN – A – 82055 – 12.

Anon., 2004. Polish Standard: Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods. PN-EN ISO 16140.

Anon., 2009. Protocol for the validation of alternative microbiological methods. NordVal. Norway.

Carter A., 1994. Using the Spearman – Karber Method to Estimate The ED50. SAS Conference Procedings, Dallas – USA, 1120–1225.

Dahlenborg M., Borch E., Rådstrom P., Prevalence of *Clostridium botulinum* types B, E and F in faecal samples from Swedish cattle. Int. J. Food Microbiol., 2003, 82, 105–110.

Fach P., Perelle F., Dilasser J., Grout C., Dargaignaratz L., Botella J. M., Gourreau F., Carlin S., Popoff M.R., Broussolle V., 2002. Detection by PCR-enzyme-linked immunosorbent assay of *Clostridium botulinum* in fish and environmental samples from a coastal area in Northern France., 68, 5870–5876.

Fenicia L., Medici D., Anibballi F., Delibato E., Aureli P., Multiplex PCR method to detect Botulinum neurotoxins producing *Clostridia* in food and clinical samples. Abstract Book of MED – VET – NET 2nd Annual General meeting 3–6 May, 2006, Malta.

Fletcher G.C., Youssef J.F., Lu G., 2008. Selecting methods for determining the presence of BoNT genes in New Zealand marine sediments. Crop & Food Research Confidential Report.

Hamilton M.A., Russo R.C., Thurston R.V., 1977. Trimmed Spearman-Karber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. Environ Sci Techn., 7, 714–719.

Hatheway C.L., Botulis, 1995. The present status of the disease. Curr. Top. Microbiol. Immunol., 195, 55–75.

Hatheway C.L., 1990. Toxigenic *Clostridia*. Clin. Microbiol. Rev. 3, 66–98.

Lindström M., Keto R., Markkula A., Nevas M., Hielm S., Korkeala H., 2001. Multiplex PCR assay for detection and identification of *Clostridium botulinum* types A, B, E, and F in food and fecal material. Appl. Environ. Microbiol., 67, 5694–5699.

Lindström M., Korkeala H., 2006. Laboratory diagnostics of botulism. Clin Microbiol Rev., 19, 298–314.

Saeed E.M.A., 2004. Studies on Isolation and Identification of *Clostridium botulinum* Investigating Field Samples Specially from Equine Grass Sickness Cases. Doctoral dissertation, Faculty of Agriculture, Goettingen, Goettingen University.

Vu T.L.A., 2006. Incidence of *Clostridium botulinum* Spores in Honey and Infant Food Samples Collected from Vietnam and Germany. Doctoral dissertation, Faculty of Agriculture, Goettingen University.

3

COMPARISON OF RFLP AND REALTIME PCR IDENTIFICATION OF C677T MUTATION IN MTHFR GENE

Introduction

Polymorphism in the MTHFR gene (5, 10 methylenetetrahydrofolate reductase) caused by mutation C677T (rs1801133) changing alanine to valine, is one of the common mutations found in the U.S. population with a frequency approximately 35% [Frosst et al. 1995, Jacques et al. 1996]. Frequency in Caucasian and Asian populations is characterized by the representation of 12 to 15% of homozygotes and about 50% heterozygotes [Ueland et al. 2001, Židek, Golian, Bulla 2011]. Since the first publication and the association of this mutation with thermolability of MTHFR enzyme [Frosst et al. 1995] relationship was analyses in fertility and diseases such as leukemia [Kamel et al. 2007, Skibola et al. 1999, Sood et al. 2010], colon cancer [Levine et al. 2010, Ma et al. 1997, WU et al. 2010], schizophrenia [Roffman et al. 2007, 2008] and many others. Individuals who are heterozygous (STs) or homozygous (TTs) for this MTHFR polymorphism have an in vitro enzyme activity that is 71 and 33% of that of (CCs) individuals, respectively [Saffroy et al. 2008]. Decreased activity of the enzyme reduces the percentage of processing of folic acid from food In subjects with the mutation (TT) is due to inadequate intake of folic acid and vitamin B12 in addition to disrupt DNA synthesis and methionine-making process and the related failure of DNA methylation. The process of DNA synthesis is essential for cell division and DNA correcting mechanisms, and its disruption leads to destabilization of the DNA and subsequent risk of chromosomal aberrations [Saffroy et al. 2004]. Inadequate methylation caused by a lack of folic acid and reduced MTHFR activity, can lead to improper cell proliferation and damage of DNA correction mechanisms and apoptosis [Friedrich et al. 2004, Jones 2001].

Relatively simple diagnosis of this mutation is carried out by RFLP method described in the works With an aspect in speed of analysis, method based on the RealTime PCR has been used. Obtained melting curve as a definition of PCR products and high resolution melting (HRM) has been used as a tool for identification of single nucleotide polymorphisms [Norambuena et al. 2009]. Melting curve analysis of PCR products (HRM) is a simple and inexpensive method suitable for mutations detecting [Reed et al. 2007, Reed and Wittwer 2004]. HRM is based on analyzing the process of DNA dissociation in the presence of dye capable of emitting radiation only in the presence of DNA double helix. The aim of this study

is to highlight the possible complications of using HRM in the diagnosis of MTHFR C677T polymorphisms based on melting curve and the proposed repeatable and accurate genotyping

Material and methods

50 samples of volunteers have been included in to comparison test with the following representation of genotypes (677CC – 16 samples, 677CT – 25 samples and 677TT – 9 samples). Restriction fragment light polymorphism has been used as a reference method for testing C677T mutation, resulting genotype was compared with the high resolution melting method based on Real Time technology.

RFLP method

Buccal mucosa has been used as a sorce material for the genetic analysis. Crude cell lysate was used as DNA donor in a volume of 2 µl. PCR reaction were performed in a total volume of 30 ml followed by using final concentrations of the ingredients: 1x GoTaq® buffer, 1,5 nmol. μl^{-1} MgCl2, 0,2 nmol. μl^{-1} dNTPs, 0,5 pmol. μl^{-1} of primers [Skibola et al. 1999] and 0,5 units of GoTaq® Hotstart polymerase The reaction was carried out in MJ Mini cyclers with an initial denaturation at 94°C for 5 minutes. Subsequent 40 cycles took place with 30 seconds denaturation at 94°C, annealing at 62°C for 30 seconds and elongation step at 72°C for 30 seconds. Final elongation of DNA was held at 71°C for 7 minutes. 10 ml of PCR products were applied to the gel in order to demonstrate specific amplification of the gene MTHFR. After the successful monitoring was 10 ml of PCR products digested with restriction endonuclease Hinf FastDigest ®.The obtained product was electrophoretically separated on 2% agarose gel at a voltage of 150 V for 45 minutes.

HRM method

PCR reaction was carried out in the LightCycler 1.5 using GoTaq ® qPCR Master Mix(Promega). Temperature regime has launched an initial denaturation at 95° C for 5 minutes. 40 cycles begins with 10 seconds denaturation at 95°C for 15 seconds, annealing at 60°C for 10 seconds and extension of DNA at 72°C for 10 seconds. The program continued to increase in temperature to 95°C for 30 seconds and cooled to 40°C for 1 minute. Melting temperature of PCR products was carried out in a temperature range of 65°C to 95°C with continuous measurement of fluorescence at a temperature increase of 0.05 °C. The resulting melting temperatures were obtained by using LightCycler Software 4.5 and evaluated in Microsoft Excel 2010.

Results

All samples were analysed using RFLP and HRM analytical approaches. HRM analysis has possibility of differentiation between the genotypes in one PCR reaction according to the melting curve of PCR products (Fig. 1). There is one peak melting curve presenting in homozygous individuals and specific double peak melting curve presented in heterozygous individuals. Double peak melting curve ensures a smooth identification of heterozygous genotypes in all samples.

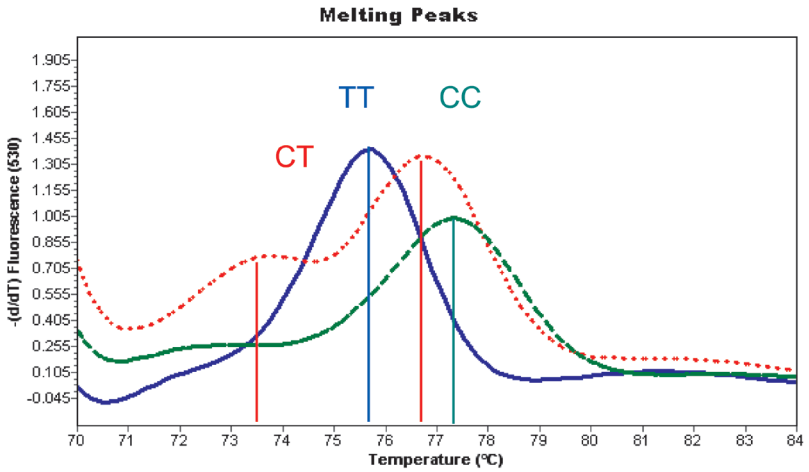


Fig. 1. Graphical representation of analysed genotypes based on the melting peaks

Average value of the melting temperature of DNA fragments without the mutation (677C) was 77.22°C, the value of the melting temperature of the fragment with a mutation (677TT) was at 76.06°C. Melting temperatures between mutated and ancestral DNA segments is thus significantly relevant difference (p=0.0018), which is usable in genotyping. Genotyping of heterozygous individual is facilitated by the presence of the second peak, located at an average of 73.74°C. However, genotyping alone is not possible only on an average heating value of the fragment, because the samples present high level of variability. Despite using the same PCR mix observed variance between the same genotypes was 0.70°C (677CC) and 0.65°C (677TT). This variability in the observed set of samples meant that the unknown sample whose melting temperature lies between 76.64°C and 78.29°C may also be assigned to genotype 677CC as well as 677TT (Fig. 2).

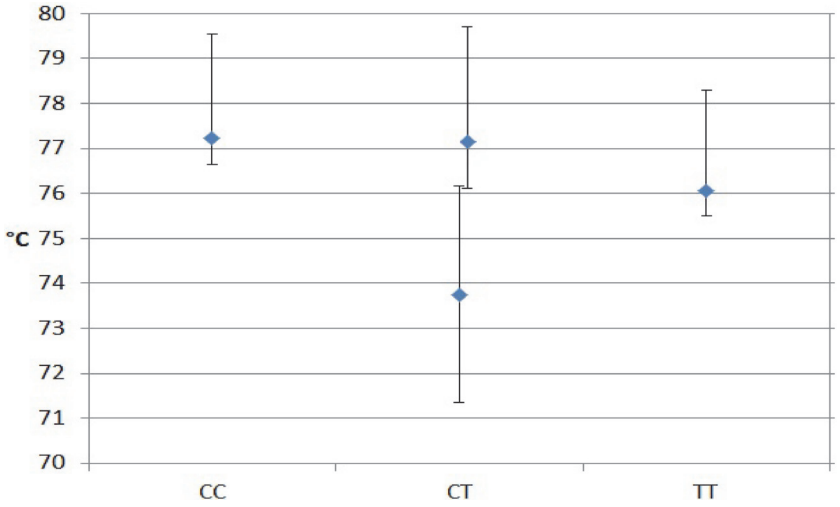


Fig. 2. Graphical representation of melting temperature variability

Environmental factors affecting the melting temperature of fragments are observable only for repeated analysis taking place in different reaction conditions. The average variation expressed in parallel samples of the same genotype is very low 0.007°C (677CC) and 0.036°C (677TT). Low variability of melting temperatures of PCR products in a single reaction condition ensures trouble-free genotyping of samples, which requires the use of control samples of known genotype for individual identification of unknown samples.

Acknowledgements

Work was supported by VEGA 1/1074/11, KEGA 3/7255/09

References

Friedrich M.G., Weisenberger D.J., Cheng J.C., Chandrasoma S., Siegmund K.D., Gonzalgo M.L. et al., 2004. Detection of methylated apoptosis-associated genes in urine sediments of bladder cancer patients. Clinical cancer research: an official journal of the American Association for Cancer Research, 10(22), 7457-65. doi: 10.1158/1078-0432.CCR-04-0930.

Frosst P., Blom H.J., Milos R., Goyette P., Sheppard C.A., Matthews R.G. et al., 1995. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nature genetics, 10(1), 111–113. Nature Publishing Group. doi: 10.1038/ng0595-111.

Jacques P.F., Bostom A.G., Williams R.R., Ellison R.C., Eckfeldt J.H., Rosenberg I.H. et al., 1996. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. Circulation, 93(1), 7–9. Retrieved June 3, 2011, from http://www.ncbi.nlm.nih.gov/pubmed/8616944.

Jones P.A., 2001. Cancer. Death and methylation. Nature, 409(6817), 141, 143–144. doi: 10.1038/35051677.

Kamel A.M., Moussa H.S., Ebid G.T., Bu R.R., Bhatia K.G., 2007. Synergistic effect of methyltetrahydrofolate reductase (MTHFR) C677T and A1298C polymorphism as risk modifiers of pediatric acute lymphoblastic leukemia. Journal of the Egyptian National Cancer Institute, 19(2), 96–105. Retrieved February 22, 2011, from http://www.ncbi.nlm.nih.gov/pubmed/19034339.

Lee H.-C., Jeong Y.-M., Lee S.H., Cha K.Y., Song S.-H., Kim N.K. et al., 2006. Association study of four polymorphisms in three folate-related enzyme genes with non-obstructive male infertility. Human reproduction (Oxford, England), 21(12), 3162–3170. doi: 10.1093/humrep/del280.

Levine A.J., Figueiredo J.C., Lee W., Conti D.V., Kennedy K., Duggan D.J. et al., 2010. A candidate gene study of folate-associated one carbon metabolism genes and colorectal cancer risk. Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 19(7), 1812–1821. doi: 10.1158/1055-9965.EPI-10-0151.

Ma J., Stampfer M.J., Giovannucci E., Artigas C., Hunter D.J., Fuchs C. et al., 1997. Methylenetetrahydrofolate Reductase Polymorphism, Dietary Interactions, and Risk of Colorectal Cancer. Cancer Res., 57(6), 1098–1102. Retrieved February 22, 2011, from http://cancerres.aacrjournals.org/cgi/content/abstract/57/6/1098.

Norambuena P.A., Copeland J.A., Krenková P., Stambergová A., Macek M., 2009. Diagnostic method validation: High resolution melting (HRM) of small amplicons genotyping for the most common variants in the MTHFR gene. Clinical biochemistry, 42(12), 1308–1316. Elsevier B.V. doi: 10.1016/j.clinbiochem.2009.04.015.

Reed G.H., Kent J.O., Wittwer C.T., 2007. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. Pharmacogenomics, 8(6), 597–608. doi: 10.2217/14622416.8.6.597.

Reed G.H., Wittwer C.T., 2004. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. Clinical chemistry, 50(10), 1748–1754. doi: 10.1373/clinchem.2003.029751.

Roffman J.L., Weiss A.P., Deckersbach T., Freudenreich O., Henderson D.C., Purcell S. et al., 2007. Effects of the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism on executive function in schizophrenia. Schizophrenia research, 92(1–3), 181–188. doi: 10.1016/j.schres.2007.01.003.

Roffman J.L., Weiss A.P., Purcell S., Caffalette C.A., Freudenreich O., Henderson D.C. et al., 2008. Contribution of methylenetetrahydrofolate reductase (MTHFR) polymorphisms to negative symptoms in schizophrenia. Biological psychiatry, 63(1), 42–48. doi: 10.1016/j.biopsych.2006.12.017.

Saffroy R., Benyamina A., Pham P., Marill C., Karila L., Reffas M. et al., 2008. Protective effect against alcohol dependence of the thermolabile variant of MTHFR. Drug and alcohol dependence, 96(1–2), 30–36. doi: 10.1016/j.drugalcdep.2008.01.016.

Saffroy R., Pham P., Chiappini F., Gross-Goupil M., Castera L., Azoulay D. et al., 2004. The MTHFR 677C > T polymorphism is associated with an increased risk of hepatocellular carcinoma in patients with alcoholic cirrhosis. Carcinogenesis, 25(8), 1443–1448. doi: 10.1093/carcin/bgh147.

Skibola C.F., Smith M.T., Kane E., Roman E., Rollinson S., Cartwright R.A. et al., 1999. Polymorphisms in the methylenetetrahydrofolate reductase gene are associated with susceptibility to acute leukemia in adults. Proceedings of the National Academy of Sciences of the United States of America, 96(22), 12810–5. Retrieved February 22, 2011, from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=23109&tool=pmcentrez&rendertype=abstract.

Sood S., Das R., Trehan A., Ahluwalia J., Sachdeva M.U., Varma N. et al., 2010. Methylenetetrahydrofolate reductase gene polymorphisms: association with risk for pediatric acute lymphoblastic leukemia in north Indians. Leukemia & lymphoma, 51(5), 928–932. doi: 10.3109/10428191003719023.

Ueland P.M., Hustad S., Schneede J., Refsum H., Vollset S.E., 2001. Biological and clinical implications of the MTHFR C677T polymorphism. Trends in pharmacological sciences, 22(4), 195–201. Retrieved June 3, 2011, from http://www.ncbi.nlm.nih.gov/pubmed/11282420.

Wu H.-C., Chang C.-H., Tsai R.-Y., Lin C.-H., Wang R.-F., Tsai C.-W. et al., 2010. Significant Association of Methylenetetrahydrofolate Reductase Single Nucleotide Polymorphisms with Prostate Cancer Susceptibility in Taiwan. Anticancer Res, 30(9), 3573–3577. Retrieved February 22, 2011, from http://ar.iiarjournals.org/cgi/content/abstract/30/9/3573.

Židek R., Golian J., Bulla J., 2011. Nutrigenomic analysis of C677T mutation of MTHFR gene in Slovak population. Potravinarstvo, 5(2), 73–76. doi: 10.5219/136.

4

APPLICATION OF ELECTROCHEMICAL DNA HYBRIDIZATION BIOSENSORS IN FOOD ANALYSIS

Introduction

The effective assurance of food safety is strictly connected with efficient evaluation of hazards arising frequently from improper preparation, storage or distribution of food raw materials and products [Luong et al. 2008]. Conventional analytical methods are often to time and labour consuming to fulfil this requirement. Among the alternative approaches introduced in food analyses there were biotechnological, molecular biology and nanoscale techniques. The last two decades provided a significant progress in development of biosensing approach exploring specific properties of DNA or other forms of nucleic acids including aptamers and peptide nucleic acids [Cheng et al. 2009, Labuda et al. 2010, Singh et al. 2010]. The biosensors were also attempted to apply in food analysis. The main directions associated with development of this analytical attitude were focused on detection of chemical compounds interacting with DNA or identification of nucleic acid fragments specific for genetically modified organisms or selected microbes [Ligaj 2010]. The second type of mentioned biosensing techniques relies on highly specific hybridization reaction between complementary DNA strands – the probe and target nucleic acid fragment. The efficient work of such kind of sensors highly depends on construction of its recognition layer and functioning of transduction system that informs about the presence of target compound in analysed sample [Tosar et al. 2010]. In the paper we describe key factors affecting DNA hybridization biosensor preparation and our experience in application of these devices in food analysis.

DNA hybridization biosensors

Biosensor is an analytical device transforming the results of biological reaction into measurable signal. Its construction bases on two principal components (Fig. 1) – detection layer comprising biologically active compounds that specifically recognize target element, and transducer providing quantifiable signal proportional to the identified characteristic. Biocompounds immobilized in biosensor detection layer are usually enzymes, antibodies, nucleic acids, tissues, cells or whole microorganisms. The transduction component can be optical,

electrochemical, piezoelectric, thermometric, magnetic or micromechanic system, as well its combination [Velusamy et al. 2010]. The work of biosensor includes three main stages [Filipiak 1999, Grieshaber et al. 2008]. At the beginning the biologically active elements of sensor detection layer specifically recognize the target compound. The physicochemical result of this reaction is further converted into measurable signal via transducer. The last step of analysis relies on processing of obtained analytical response, its amplification and/or recording.

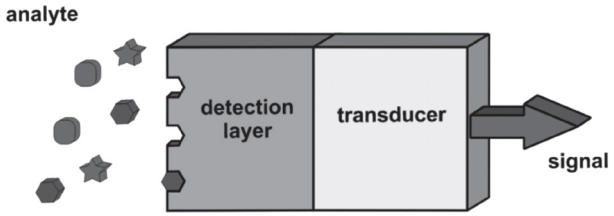


Fig. 1. Scheme of biosensor construction [Filipiak 1999]

Taking into consideration the practical usability and potential application in miniaturized analytical systems the most perspective type of transducer in sensors is electric array. More than a half of described in the literature biosensors relies on electrochemical signal transduction in respect of its high sensitivity, simplicity in construction and inexpensive use [Luong et al. 2008]. Additionally electrochemical sensors require usually only a small amount of analysed substances and they can be used in case of turbid samples [Grieshaber et al. 2008].

DNA hybridization biosensors are commonly connected with electrochemical transducers that are applied most often in a form of solid working electrodes. Their construction relies on immobilization on the surface of transducer (electrode) a layer of single stranded nucleic acid compounds (ssDNA probes) that are complementary to target DNA fragment (Fig. 2). ssDNA probe contains usually from 15 to 30 nucleotides and its sequence cannot predetermine formation of any structures between themselves [Palecek and Fojta 2005]. The detection of specific nucleic acid fragments is carried out by immersion of biosensor detection layer into sample containing analysed DNA. In the presence of target DNA fragments (complementary to ssDNA probes) a hybridization reaction proceeds and double stranded DNA duplex (dsDNA) is formed on the transducer surface [Labuda et al. 2010]. Usually the hybridization event and further detection of its results are performed in biosensor detection layer – it is a type of one surface technique. Another attitude called "sandwich array" is applied when recognition of target DNA and detection of newly formed dsDNA are proceeded in two separate assemblies [Palecek and Fojta 2005].

After the interaction of ssDNA probes with analysed nucleic acid samples whole attention is focused on the identification of the results of this action. The main goal is then verification whether the biosensor detection layer contains double stranded nucleic acid fragments or not [Palecek and Fojta, 2005]. The identification of dsDNA compounds in electrochemical biosensor can be performed directly or requires application of some additional reagents such hybridization indicators or substrates for enzymatic reaction. More complex techniques are connected also with chemical modification of probes or target nucleic acid fragments [Lucarelli et al. 2008]. The most popular attitudes applied in electrochemical hybridization biosen-

sors base on natural electric activity of nucleic acids or use of electroactive redox indicators that bind specifically to double or single stranded DNA [Labuda et al. 2010].

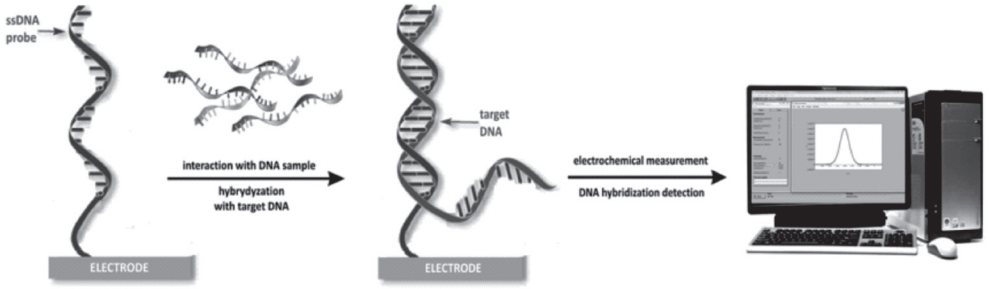


Fig. 2. Scheme of electrochemical biosensor for detection of nucleic acid hybridization

All nucleic acid bases are able to electrochemical oxidation but for analytical purpose the most applicable are electroactive properties of guanine [Stempkowska et al. 2007]. Theoretically its electrochemical signal should be enhanced in case of successful hybridization event (and formation DNA duplexes) in biosensor detection layer as a result of guanine amount increase. In practise there are some disadvantageous factors that complicate such direct attitude of hybridization detection. Guanine moieties in double stranded DNA undergoes an oxidation with higher difficulty than bases in ssDNA fragments [Ligaj 2010]. Additionally the electrochemical signal of guanine depends on its distance from the electrode surface [Labuda et al. 2010]. One of the methods to simplify this direct hybridization detection is replacement of guanine in ssDNA probes with inosine compound [Kerman et al. 2003]. Inosine binds also specifically with cytosine (similar to complementarity principle between nucleic acid bases) but it is threefold less electroactive than substituted guanine. Thanks to the replacement hybridization can be detected via measuring the electrochemical response provided by guanine moieties present only in target nucleic acid fragments immobilized in biosensor detection layer [Ligaj 2010].

The identification of hybridization results obtained on sensor surface can be quite easily performed with the use of electroactive indicators that noncovalently bind nucleic acids. These chemical compounds show different affinity for single or double stranded DNA [Palecek and Fojta 2005]. The indicator application relies usually on comparison of the scale of its accumulation in biosensor detection layer before and after the interaction of ssDNA probes with analysed nucleic acid sample [Dharuman and Hahn 2007]. The amount of accumulated indicator in preferred DNA form is much significant and the measurement of its electrochemical signal enabled quantification of target nucleic acid compounds [de-los-Santos-Alvarez et al. 2004].

The noncovalent binding between indicator and DNA can rely on electrostatic interactions, minor or major groove binding in dsDNA structure as well on intercalation of double helix of nucleic acids. These interactions are driven with van der Waals forces, hydrogen or hydrophobic bonds and/or reversible charge exchange [Li et al. 2007]. Metal complexes such as hexaammineruthenium (III) ($[Ru(NH_3)_6]^{3+}$) or tris(2,2'-bipyridyl) cobalt (III) ($[Co(bpy)_3]^{3+}$) ions are able to quantitative electrostatic binding to negatively charged deoxiribose-phosphate backbone of DNA fragments (both single and double stranded). Evaluation of metal complex amount that is accumulated in biosensor detection layer enable the estimation

of DNA surface density and/or hybridization efficiency – on the basis of DNA bounded on the electrode surface [Ligaj et al. 2008, Wong et al. 2005]. The much higher specificity in discrimination between single and double stranded nucleic acids is revealed with intercalating compounds. This kind of indicators has often a planar structure with aromatic rings that facilitates penetration (intercalation) of this compounds between paired nucleic acid bases in double stranded DNA [Li et al. 2007, Palchaudhuri and Hergenrother 2007]. The intercalator accumulates more efficiently on electrode surface containing immobilized dsDNA (after successful hybridization event) than in biosensor detection layer consisted only from ssDNA probes [Labuda et al. 2010]. In electrochemical biosensors for hybridization detection quite common were applied such intercalating indicators like daunomycin [Kobayashi et al. 2004, Lee et al. 2007], ethidium bromide [Kobayashi et al. 2004, Liu et al. 1997] or anthraquinone-1,5-disulfonic acid disodium salt [Wong et al. 2005]. Another mechanism of noncovalent interaction with DNA is presented by methylene blue (MB) – an organic dye from phenothiazine family. This electroactive indicator reveals a strong affinity for guanine moieties in single stranded nucleic acid fragments [Yang et al. 2002]. Successful hybridization event confines the accumulation of MB in biosensor detection layer and therefore the electrochemical response of the indicator is lower [Jin et al. 2007, Tichoniuk et al. 2008]. The most specific dsDNA identification is provided with chemical compounds that interact with DNA in places called grooves – structures typical only for double stranded nucleic acids [McEwen et al. 2008]. Such indicators bind dsDNA in its small grooves with different type intermolecular interactions. A good example of groove binding indicator is benzimide dye Hoechst 33258 that was used in several DNA hybridization sensors [Choi et al. 2006, Kobayashi et al. 2004].

Key factors affecting DNA biosensor preparation

Although idea of biosensors' construction is quite simple there are some critical points in these devices preparation and their application in food sample analysis. Crucial role plays a proper selection of ssDNA probes sequence and further the way of its immobilization in sensor detection layer. The sequence of probe should be both highly specific for target nucleic acid fragment and fulfil the requirement associated directly with its construction. The selected DNA fragments (usually synthetic oligonucleotides with 15 to 30 base pairs) are evaluated regarding its ability to form secondary structure (so-called "hairpin structure") and dimmers [Lucarelli et al. 2008]. The exclusion of oligonucleotides transforming into such structures can be easily made using DNA calculators like Sigma-Genosys [Sigma-Genosys 2011]. The specificity of ssDNA probes towards the target nucleic acid fragmentis commonly assessed with the help of BLAST software (Basic Local Alignment Search Tool) that makes fast and reliable analysis of the sequences collected in GenBank database [Sayers et al. 2009].

The method of ssDNA probes immobilization in biosensor detection layer must enable its unlimited interaction with target nucleic acid fragments in optimized conditions of this reaction i.e. pH value, temperature, ionic strength, etc. [Labuda et al. 2010]. The simplest approach of probe immobilization on solid electrode surface bases on electrostatic interactions between negatively charged deoxiribose-phosphate backbone of DNA and positively polarized electrode surface [de-los-Santos-Alvarez et al. 2004]. Through its simplicity this method can unfortunately lead to the limitation of DNA specific properties, its unstable connection

with electrode or less effective hybridization with target nucleic acids. Much better results in ssDNA probe immobilization in biosensor detection layer are obtained using so-called one--point attachment techniques often combined with formation of covalent bounds. One-point binding of nucleic acid strands (at its 5' or 3' end) enables strong DNA immobilization on the transducer surface and simultaneously assures its conformational freedom [Arya et al. 2009]. The covalent bounds between ssDNA probes and electrode can be formed modifying transducer surface with chemically active groups that with the use proper activating agents (e.g. carbodiimide derivatives) create strong connection with one of DNA molecule ends [Ligaj 2010]. Similar procedure is applied in electrochemical biosensors performed on the basis of gold electrodes modified with organothiolated self-assembled monolayers (SAM). Strong interaction between sulphur and gold atoms provides covalent immobilization of molecules on the electrode [Tichoniuk and Filipiak 2008]. Modifying one of ssDNA probe end with thiolated alkyl linker it is possible its binding on the gold electrode surface and creating a self-assembled monolayer of single stranded nucleic acids [Wong et al. 2005].

Thiolated ssDNA probes are quite frequently used in electrochemical DNA biosensors for hybridization detection because of uncomplicated procedure of its stable and ordered immobilization in SAM monolayer on gold electrodes [Tichoniuk and Filipiak 2008]. They are usually applied in mixed self-assembled layers in connection with mercaptoalcohol compounds (so-called diluent) that create packing of newly formed SAM [Wong et al. 2005, Arya et al. 2009]. Such assembly can provide desirable organization of biosensor detection layer (Fig. 3) with fully accessible ssDNA probes. Additionally mercaptoalcohol compounds such like mercaptohexanol could protect electrode surface from non-specific adsorption of molecules other than target nucleic acid fragments [Henry et al. 2010]. The alcoholic groups of diluent compounds repel also negatively charged DNA backbones and oriented them into the analysed solution in the close vicinity of biosensor detection layer [Dharuman et al. 2007, Tichoniuk et al. 2010].

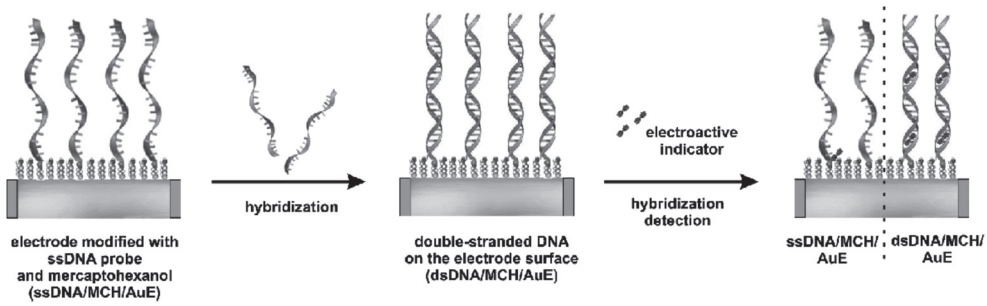


Fig. 3. Application of mixed SAM and electroactive indicators in electrochemical DNA biosensor functioning [Tichoniuk et al. 2010]

The satisfactory work of electrochemical DNA biosensor depends also on effective and reliable system of hybridization detection. A lot of benefits comes with the application of electroactive indicators that can efficiently differentiate single and double-stranded nucleic acids present in biosensor detection layer [Labuda et al. 2010]. After the interaction ssDNA probes with analysed nucleic acid sample biosensing interface is usually immersed in solution containing such indicator that specifically accumulate in single or double stranded DNA molecules (Fig. 3) [Tichoniuk et al. 2010]. From practical point of view the selected electro-

active indicator should provide redox potential of its electrochemical response in the range distinctively different than the values connected with nucleic acid bases oxidation (usually there are lower potential values) [Ligaj 2010]. The proper choice of indicator enabled effective monitoring of hybridization proceeded in biosensor detection layer and it should not be disturbed with electric activity of other substances present in analysed sample [Palecek and Fojta 2005].

The detection of genetically modified (GM) food compounds

Identification of genetically modified food compounds bases generally on the detection of specific for GMO proteins (mainly using immunological tests) or nucleic acid fragments. The procedures focused on the recognition of the second biomolecules are dominated with techniques exploring polymerase chain reaction (PCR) [Holst-Jensen 2009]. Despite the high specificity and sensitivity of this attitude there is still a need of other alternative tools for GM food compounds detection that simplify and cut down the time of such analyses [Ligaj 2010]. Following this trend many DNA hybridization biosensors were presented in the literature in the recent years.

One of the popular approaches are piezoelectric sensors designed for the identification of nucleic acid fragments specific for regulatory segments of introduced DNA or directly connected with the desired feature implemanted into GM organism. For example it was constructed a biosensor capable to detect fragment of *cryIA(b)* gene that provides genetically modified maize resistance to insects [Passamano and Pighini 2006]. Other piezoelectric sensors were designed to identify a fragment of ESPS gene (responsible for Roundup Ready herbicide resistance of RR soybean) [Stobiecka et al. 2007, Bogani et al. 2009] or P35S promoter (often present in genetically modified plants) [Karamollaoglu et al. 2009]. In all presented biosensors a quartz microbalance array responds positively with the change in its oscillation frequency in case of successful hybridization event between ssDNA probes (immobilized on microbalance pan) and target DNA fragments.

Typical electrochemical biosensor devoted to detection of DNA fragment specific for *bar* gene (encoding phosphinothricin herbicide resistance) was performed by our research group on the basis of chemical modification of carbon paste electrode (CPE) surface [Ligaj et al. 2008]. ssDNA probes were covalently attached by 5'phosphate end to carboxyl groups of stearic acid introduced into carbon paste outer layer. In construction of the biosensor detection layer carbodiimide derivatives i.e. 1-ethyl-3(3′-dimethylaminopropyl)-carbodiimide and N-hydroxy-sulfosuccinimide were used as activating agents. The detection of hybridization reaction at the electrode surface was performed via direct voltammetric measurement of guanine electrochemical response and evaluation of accumulation rate of tris (2,2'-bipyridyl) cobalt (III) ($[Co(bpy)_3]^{3+}$) complex as an electroactive indicator. In the first situation the obtained detection limit equalled 1 μM of target DNA but it has quite low reproducibility. The cobalt complex $[Co(bpy)_3]^{3+}$ provided much better repeatability of the results and significantly lower limit of detection – 0,1 μM. Complete time of measurement with the biosensor application took about 70 minutes (including detection layer preparation, hybridization with target DNA and interaction with electroactive indicator).

Other our biosensing project were focused on preparation of electrochemical DNA biosensor for GMO detection using gold electrodes modified with self-assembled layer consisted

from cysteamine (linking compound) and ssDNA probes [Tichoniuk et al. 2008]. Immobilized oligonucleotide probes were complementary to target nucleic acid fragments specific for P35S promoter or *Tnos* terminator (typical regulatory sequences introduced into GM plants).

The results of analysed hybridization reaction were evaluated with voltammetric measurement of indicator methylene blue. MB molecules were less effectively accumulated in biosensor detection layer containing double stranded DNA. Indicator voltammetric signal was then significantly lower (Fig. 4A and 4B). Both applied ssDNA probes enabled distinct differentiation of the nucleic acid samples isolated from RounddupReady® (RR) soybean and non-genetically modified (non-GM) soybean. Voltammetric signal of methylene blue in reference measurements with DNA isolated from non-GM soybean was similar to the indicator response accumulated before biosensor interaction with analysed DNA sample. They equalled 0.191 μA (RSD 15%) and 0.233 μA (RSD 16%) (before interaction) for P35s gene fragment identification, 0.351 μA (RSD 7%) and 0.339 μA (RSD 6%) for *Tnos* gene fragment analyses respectively

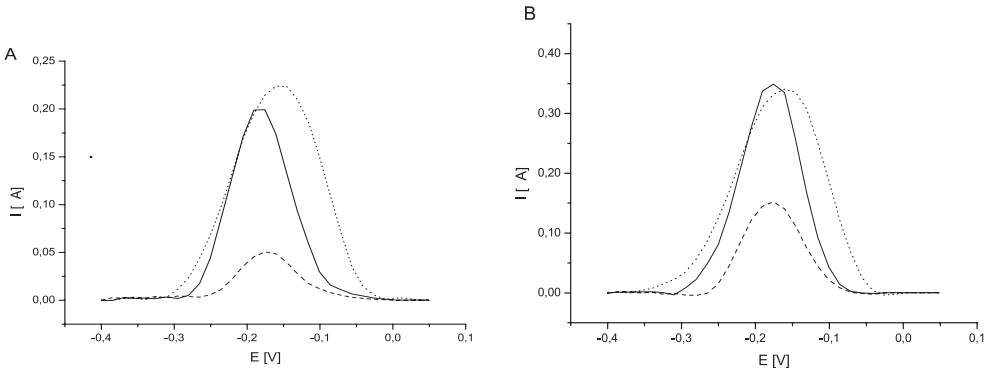


Fig. 4. Voltammograms of methylene blue molecules accumulated in the biosensor detection layer containing ssDNA probes specific for P35S promoter (A) or *Tnos* terminator (B) before interaction with analysed DNA sample (dotted line) and after reaction with DNA isolated from RoundupReady (dashed line) or non-GM soybean (solid line) – both in concentration of 1.5 μg /ml

The successful hybridization of ssDNA probes with target nucleic acid fragments in biosensor detection layer led to the significant decrease of methylene blue accumulation and reduction of its voltammetric response. The MB signal was lowered to the value of 0,049 μA (RSD 14%) in case of P35s promoter gene fragment identification and to 0,151 μA (RSD 5%) for *Tnos* terminator gene fragment (Fig. 4A and 4B). Presented biosensing arrays were applied to detect nucleic acid sequences that are used in wide variety of GMOs and the sensor could be adapted to the identification of other genetically modified food components.

Foodborne pathogen detection

Detection of pathogenic microorganisms contaminating food products might be performed with very different analytical tools that can be grouped according with the microbial characteristics evaluated during the identification. They are usually divided into

bio- or immunochemical methods, phenotypic or genetic ones [Lazcka et al. 2007, Goderska and Szwengiel 2009]. Conventional techniques evolved from decades have now status of reference procedures for the foodborne pathogen detection [Jasson et al. 2010]. Despite some their limitations they offer highly specific and sensitive analyses of microbial hazard [Velusamy et al., 2010]. The main disadvantage connected with conventional methods of foodborne pathogen detection is their time and labour consuming performance. Preliminary results of the microbiological identification are given usually in 2–3 days. Its confirmation takes from 7 to 10 days [Montville and Matthews 2008].

The proposed alternative approaches of pathogenic microorganisms detection aim at faster and reliable evaluation of this health hazard. One of developed attitudes are biosensors capable to identify a specific nucleic acid fragments in analysed food sample [Velusamy et al. 2010]. The bacterium associated usually with microbial contamination of food *Escherichia coli* is the detection target of many biosensors presented lately in the literature. One of them was constructed using air-plasma-activated fullerene-impregnated screen printed electrodes [Shiraishi et al. 2007]. The immobilized on the transducer surface ssDNA probes were specific to 16S RNA fragment that was present in nucleic acid sample isolated from *E. coli* cells. The hybridization of target DNA in the sensor detection layer was evaluated by voltammetric measurements of electroactive indicator tris(2,2'-bipyridyl) cobalt (III) complex. Other biosensors devoted to the detection of nucleic acid fragments specific for *E. coli* via its hybridization with ssDNA probes based either on electrochemical [Loaiza et al. 2007] or piezoelectric transducers [Chen et al. 2007].

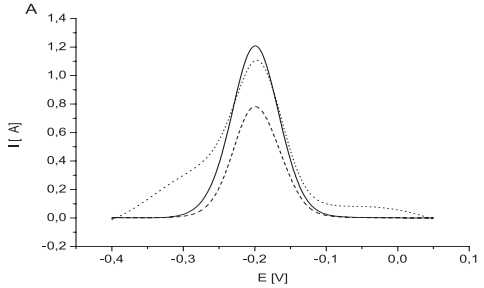
The first electrochemical biosensor for recognition of DNA fragments specific for foodborne pathogen, that was constructed in our laboratory, was designed to the identification of *Listeria monocytogenes* [Oczkowski and Filipiak, 2009]. The sensor detection layer was obtained by the modification of carbon paste electrode with ssDNA probes specific for *hlyA* gene fragments (typical for *L. monocytogenes*). After the hybridization with target nucleic acids the results of this reaction was assessed by voltammetric measurement of electroactive indicator daunomycin. Its compounds preferentially binds to double stranded DNA and its higher accumulation rate in biosensor detection layer demonstrated the presence of target DNA fragments in analysed sample.

Our last research work was concentrated on preparation of electrochemical biosensor for detection of nucleic acid fragments specific for aerolysine gene, toxin secreted by *Aeromonas hydrophila* [Tichoniuk et al. 2010]. This bacterium is classified to emerging pathogens group. It could easily colonize low-proceeded food products stored in refrigerating conditions.

The biosensor recognition layer based on chemical modification of gold electrodes with mixed self-assembled monolayer (SAM) that consisted of thiolated ssDNA probes and mercaptohexanol compounds (diluent molecules). The construction of the sensor detection interface was thoroughly examined using voltammetric evaluation of its double layer capacitance and chronocoulometric quantitation of DNA present on the electrode surface [Tichoniuk et al., 2010]. Recognition of target nucleic acid fragments specific for aerolysin gene relied on hybridization reaction between analysed DNA sample and ssDNA probes immobilized in sensor detection layer. The monitoring of the results of this interaction was performed using voltammetric measurements of methylene blue accumulated on the gold electrode surface (Fig. 5A). The electrochemical biosensor enabled successful differentiation between DNA samples isolated from water contaminated with *A. hydrophila* and other bacteria. Voltammetric signal of methylene blue accumulated after successful hybridization event significantly

decreased in comparison with indicator response in reference experiments and confirmed the formation of nucleic acid duplexes in sensor detection layer. DNA sample isolated from water contaminated by other than *A. hydrophila* microorganism neither participated in hybridization reaction nor interacted non-specifically to the gold electrode surface [Fig. 5B].

A



B

type of analysis	the signal of accumulated methylene blue [μA] (RSD [%])
before interaction with analysed DNA sample	1.12 (14%)
after interaction with DNA of A. *hydrophila*	0.78 (8%)
after interaction with reference becterial DNA	1.25 (21%)

Fig. 5. Voltammograms of MB indicator molecules accumulated in the biosensor detection layer containing ssDNA probes specific for aerolysin gene before interaction with analysed DNA sample (dotted line) and after reaction with DNA of *A. hydrophila* (dashed line) or reference bactetial DNA (A) and the comparison of this measurements results (B)

Conclusions

DNA hybridization biosensors are devices attempted to apply in identification of nucleic acid fragments typical for selected food components (e.g. genetically modified additives) or indicating food pollution such as microbial or pathogenic one. Electrochemical systems of hybridization recognition provide promising attitude in sensors' development in relation to its abilities in fast, simple and inexpensive application. The performance of effective DNA hybridization sensor requires precise selection of ssDNA probes sequence, the construction of biosensing interface that saves its natural specificity and reliable system of DNA duplex identification. All these goals we tried to achieve in our work on electrochemical biosensors. We constructed devices able to detect nucleic acids fragments specific for genetically modified food compounds and typical for microorganisms that can be hazardous for consumers. Although the presented biosensors have some drawbacks they are still very promising tools in alternative attitudes for food safety control and they deserve for their further development.

Acknowledgements

This work was financially supported by the Polish Ministry of Science and High Education. Grant No. N N312 206736

References

Arya S.K., Solanki P.R., Datta M., Malhotra B.D., 2009. Recent advances in self-assembled monolayers based biomolecular electronic devices. Biosens Bioelectron, 24, 2810–2817.

Bogani P., Minunni M., Spiriti M.M., Zavaglia M., Tombelli S., Buiatti M., Mascini M., 2009. Transgenes monitoring in an industrial soybean processing chain by DNA-based conventional approaches and biosensors. Food Chem., 113, 658–664.

Chen S.H., Wu V.C.H., Chuang Y.Ch., Lin Ch.S., 2008. Using oligonucleotide-functionalized Au nanoparticles to rapidly detect foodborne pathogens on a piezoelectric biosensor. J. Microbiol Meth., 73, 7–17.

Cheng A.K.H., Sen D., Yu H.Z., 2009. Design and testing of aptamer-based electrochemical bio-sensors for proteins and small molecules. Bioelectrochemistry, 77, 1–12.

Choi Y.S., Lee K.S., Park D.H., 2006. Gene detection using Hoechst 33258 on a biochip. Curr. Appl. Phys., 6, 777–780.

de-los-Santos-Alvarez P., Lobo-Castanon M.J., Miranda-Ordleres A.J., Tunon-Blanco P., 2004. Current strategies for electrochemical detection of DNA with sold electrodes. Anal Bioanal Chem., 378, 104–118.

Dharuman V., Hahn J.H., 2007. Effect of short chain alkane diluents on the label free electrochemical DNA hybridization discrimination at the HS-ssDNA/diluent binary mixed monolayer in presence of cationic intercalators. Sensor Actuator., 127, 536–544.

Filipiak M., 1999. Wykorzystanie substancji biologicznie czynnych do celów analitycznych w biosensorach enzymatycznych i biosensorach DNA, [w:] Koroniak H., Barciszewski J. (Ed.) Na pograniczu chemii i biologii. Tom III. Wydawnictwo Naukowe UAM Poznań, 481–501.

Goderska K., Szwengiel A., 2009. Nowe metody stosowane w analizie żywności. Laboratorium, 5, 10–15.

Grieshaber D., MacKenzie R., Voros J., Reimhult E., 2008. Electrochemical biosensors – sensor principles and architectures. Sensors, 8, 1400–1458.

Henry O.Y.F, Gutierrrez Perez J., Acero Sanchez L.J., O'Sullivan C.K., 2010. Electrochemical characterisation and hybridisation efficiency of co-assembled monolayers of PEGylated ssDNA and mercaptohexanol on planar gold electrodes. Biosens Bioelectron., 25, 978–983.

Holst-Jensen A., 2009. Testing for genetically modified organisms (GMOs): Past, present and future perspectives. Biotechnol Adv., 27, 1071–1082.

Jasson V., Jacxsen L., Luning P., Rajkovic A., Uyttendaele M., 2010. Alternative microbial methods: An overview and selection criteria. Food Microbiol., 27, 710–730.

Jin Y., Yao X., Liu Q., Li J., 2007. Hairpin DNA probe based electrochemical biosensor using methylene blue as hybridization indicator. Biosens Bioelectron., 22, 1126–1130.

Karamollaoglu I., Oktem H.A., Mutlu M., 2009. QCM-based DNA biosensor for detection of genetically modified organisms (GMOs). Biochem Eng J., 44, 142–150.

Kerman K., Morita Y., Takamura Y., Tamiya E., 2003. Label-free electrochemical detection of DNA hybridization on gold electrode. Electrochemi Commun., 5, 887–891.

Kobayashi M., Kusakawa T., Saito M., Kaji S., Oomura M., Iwabuchi Sh., Morita Y., Hasan Q., Tamiya E., 2004. Electrochemical DNA quantification based on aggregation induced by Hoechst 33258. Electrochem Commun., 6, 337–343.

Labuda J., Oliveira Brett A.M., Evtugyn G., Fojta M., Mascini M., Ozsoz M., Palchetti I., Palecek E., Wang J., 2010. Electrochemical nucleic acid-based biosensors: Concepts, terms, and methodology (IUPAC Technical Report). Pure Appl Chem., 82, 1161–1187.

Lazcka O., Del Campo F.J., Munoz F.X., 2007. Pathogen detection: A perspective of traditional methods and biosensors. Biosens Bioelectron., 22, 1205–1217.

Lee J.G., Yun K., Lim G.S., Lee S.E., Kim S., Park J.K., 2007. DNA biosensor based on the electrochemiluminescence of $Ru(bpy)_3^{2+}$ with DNA-binding intercalators. Bioelectro-chemistry., 70, 228–234.

Li H.H., Aubrecht J., Fornace Jr. A.J., 2007. Toxicogenomics: Overview and potential applications for the study of non-covalent DNA interacting chemicals. Mutat Res., 623, 98–108.

Ligaj M., Tichoniuk M., Filipiak M., 2008. Detection of bar gene encoding phosphi-nothricin herbicide resistance in plants by electrochemical biosensor. Bioelectrochemistry, 74, 32–37.
Ligaj M., 2010. Bioczujniki do wykrywania GMO. Wydawnictwo Uniwersytetu Ekonomicznego w Poznaniu.
Liu S., Ye J., He P., Fang Y., 1997. Voltammetric determination of sequence specific DNA by electroactive intercalator on graphite electrode. Anal. Chim. Acta, 335, 239–243.
Loaiza O.A., Campuzano S., Pedrero M., Pingarron J.M., 2007. DNA sensor based on an *Escherichia coli lac* Z gene probe immobilization at self-assembled monolayers-modified gold electrodes. Talanta., 73, 838–844.
Lung J.H.T., Male K.B., Glennon J.D., 2008. Biosensor technology: Technology push versus market pull. Biotechol Adv., 26, 492–500.
Lucarelli F., Tombelli S., Minunni M., Marrazza G., Mascini M., 2008. Electrochemical and piezoelectric DNA biosensors for hybridisation detection. Anal. Chim. Acta, 609, 139–159.
McEwen G.D., Chen F., Zhou A., 2009. Immobilization, hybridization, and oxidation of synthetic DNA on gold surface: Electron transfer investigated by electrochemistry and scanning tunneling microscopy. Anal. Chim. Acta, 643, 26–37.
Montville T.J., Matthews K.R., 2008. Food Microbiology. An Intruduction. Washington, USA: ASM Press.
Oczkowski T., Filipiak M., 2009. Startery, elektrochemiczny biosensor DNA oraz sposób wykrywania mikroorganizmu *Listeria monocytogenes* w substancjach organicznych, zwłaszcza żywności. Patent PL 200797 B1.
Palchaudhuri R., Hergenrother P.J., 2007. DNA as a target for anticancer compounds: methods to determine the mode of binding and the mechanism of action. Curr Opin Biotech., 18, 497–503.
Palecek E., Fojta M., 2005. Electrochemical DNA Sensors. w Willner, I., Katz, E. (Ed.) Bioelectronics. From Theory to Applications. WILEY-VCH Verlag GmbH & Co, Weinheim, Niemcy, 127–192
Passamano M., Pighini M., 2006. QCM DNA-sensor for GMOs detection. Sensors Actuat B-Chem., 118, 177–181.
Sayers E.W., Barrett T., Benson D.A., Bolton E., Bryant S.H., Canese K., Chetvernin V., Church D.M., DiCuccio M. et al., 2009. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res., 38, D5–D16.
Shiraischi H. Itoh T., Hayashi H., Takagi K., Sakane M., Mori T., Wang J., 2007. Electrochemical detection of E. coli 16S rDNA sequence using air-plasma-activated fullerene-impregnated screen printed electrodes. Bioelectrochemistry, 70, 481–487.
Sigma-Genosis. data dostępu: 2011. http://www.sigma-genosys.com/calc/DNACalc.asp
Singh R.P., Oh B.K., Choi J.W., 2010. Application of peptide nucleic acid towards development of nanobiosensor arrays. Bioelectrochemistry, 79, 153–161.
Stempkowska I. Ligaj M., Jasnowska J., Langer J., Filipiak M., 2007. Electrochemical response of oligonucleotides on carbon paste electrode. Bioelectrochemistry, 70, 488–494.
Stobiecka M., Cieśla J.M., Janowska B., Tudek B., Radecka H., 2007. Piezoelectric sensor for determination of genetically modified soybean Roundup Ready in samples not amplified by PCR. Sensors, 7, 1462–1479.
Tichoniuk M., Filipiak M., 2008. Wykorzystanie samoorganizujących się monowarstw organo-tiolowych w badaniach struktur biologicznych i biodetekcji, [in:] Koroniak H., Barciszewski J. (Ed.) Na pograniczu chemii i biologii. Tom XX. Poznań: Wydawnictwo Naukowe UAM, 561–579.
Tichoniuk M., Gwiazdowska D., Ligaj M., Filipiak M., 2010. Electrochemical detection of foodborne pathogen *Aeromonas hydrophila* by DNA hybridization biosensor. Biosens. Bioelectron., 26, 1618–1623.
Tichoniuk M., Ligaj M., Filipiak M., 2008. Application of DNA hybridization biosensor as a screening method for the detection of genetically modified food components. Sensors, 8, 2118–2135.

Tosar J.P., Branas G., Laiz J., 2010. Electrochemical DNA hybridization sensors applied to real and complex biological samples. Biosens. Bioelectron., 1205–1217.

Velusamy V., Arshak K., Korostynska O., Oliwa K., Adley C., 2010. An overview of foodborne pathogen detection: In the perspective of biosensors. Biotechnol Adv., 28, 232–254.

Wong E.L.S., Chow E., Gooding J.J., 2005. DNA recognition interfaces: The influence of interfacial design on the efficiency and kinetics of hybridization. Langmuir., 21, 6957–6965.

Yang W., Ozsoz M., Hibbert D.B., Gooding J.J., 2002. Evidence for the direct interaction between methylene blue and guanine bases using DNA-modified carbon paste electrodes. Electroanal., 14, 1299–1302.

5

APPLICATION OF IMPEDIMETRIC METHOD TO QUANTITATIVE EVALUATION OF LISTERIA MONOCYTOGENES CELLS IN FETA CHEESE DURING STORAGE

Introduction

A foodstuff contamination by pathogenic bacteria and their toxins poses a hazard of infections to consumers. The presence of pathogens in foodstuffs is mostly related to the quality of raw materials, and selection of food processing and preserving methods. Occurrence of microbiological hazard can result from the presence of microorganisms in the raw material due to its primary as well as secondary contaminations caused by storage and transport conditions.

Maintaining cleanliness of devices, working surface, and packaging, as well as the usage of the proper control methods of microbiological quality is a warranty of quality and stability of the foodstuffs [Windyga and Scieżyńska 2010].

During risk assessment, bacteria of *Listeria monocytogenes* species are considered currently as the main agent of the risk of development of pathogenic bacteria in food products during their production and storage in cooling conditions. This is a Gram-positive rod-shaped bacterium, which tolerates a broad range of temperature (1–45°C), and is widely spread in the environment. *Listeria monocytogenes* causes listeriosis, a serious infection for people with impaired immune system and is found with a frequency of 10 cases per 1 million populations.

However, all recorded epidemic outbreaks mostly resulted from consumption of cheeses, milk, vegetables, and meat. In Europe, around 4% of cheese products is contaminated with bacteria of *L. monocytogenes* species according to Ryser [2002]. These contaminations concern mainly soft and semi-soft cheeses, which surface aging with the participation of molds and bacteria. This pathogenic bacteria is vary rarely detected in a long aging cheeses (like Cheddar) and cheeses, such as Cottage cheese and Mozzarella, which were subjected to thermal processing during production process [Kowalik et al. 2009].

To improve and increase the guarantee of food safety, Good Manufacturing Practice (GMP) and Good Hygienic Practice (GHP) as well as Hazard Analysis and Critical Control Points (HACCP) were implemented into the food chain. Currently, there is a need to develop solutions, which enable to integrate safety programs with their predictable effect on consu-

mer health. One of the effective tools supporting a hazard risk assessment of process of food production and distribution safety is predictive microbiology (Regulation EC No 178/2002).

Providing safe, healthy food is the high priority for food technologists. The presence of hazardous pathogens in food, such as *Listeria monocytogenes*, can pose a real risk for human health or even life. The PN-EN ISO 22000:2006 standard indicates on necessity to provide safe, healthy food on every stage of the process of food production and distribution – "from the agriculture field to the end consumer" [Kijowski et al. 2008]. Currently, Regulation (EC) No 2073/2005 (15th November, 2005) represents the effective legal act on microbiological contamination of food. This particular directive includes microbiological criteria for foodstuffs. This regulation contains two kinds of microbiological criteria:

- food safety criterion – means the level of microbiological contaminations defining the acceptability of a product to be placed on the market or already being in the distribution,
- process hygiene criterion – a criterion indicating the acceptable functioning of the production process. Such a criterion is not applicable to products placed on the market.

Safety food criteria relate to the presence of pathogenic bacteria (*Listeria monocytogenes, Salmonella sp., Escherichia coli, Enterobacter sakazakii*) and staphylococcal enterotoxin and histamine in foodstuffs."

Regulation (EC) No 2073/2005 regulates among others the problem of contamination of foodstuffs by *Listeria monocytogenes*. To provide consumer protection, the foodstuffs were divided into two categories:

1. food intended for newborns, for special medical care, and ready-to-eat food in which growth of a high number of these bacteria is possible,
2. food in which *L. monocytogenes* can not proliferate [Nowak 2007].

The safety of the food production process is related to the analysis and microbiological risk assessment (supporting by tools of predictive microbiology). Having into consideration the consumer safety, the hazard risk assessment is based on thorough analysis of all possible risks that can occur and then searching for all possible ways to eliminate or diminish sizes of the considered risk [Maćkiw and Szponar 2003, Mafart 2005].

Alternative methods of microbiological analyses

Prolonged time of analysis and significant amount of materials and costs accompany evaluation of microbiological quality of foodstuffs with the use of classical methods, such as plate methods. In the case of systems like HACCP, there is a need for fast risk assessment and recommendation of specific critical value below or above which the safety of production process is endangered. Therefore, a fast and effective analysis, which provides specific and valid data, is very significant. One of the ways to cut the time of analysis is application of impedimetric method in predictive microbiology [Łobacz et al. 2008]. There are many impedimetric systems for microbiological analysis, such as Malthus instrument (Malthus Instruments Ltd, Wielka Brytania); Rabit (Don Whitely Scientific Ltd, Wielka Brytania); Bactometer (bioMerieux, Wielka Brytania); Honeywell-Wade System (Honeywell Inc., Littleton, USA); Orega 6 Automatic Microbial Analyzer (Japan Tectron Instrument Corpo-

ration); BacTrac (Sy-lab, Austria) [Czajkowska and Witkowska-Gwiazdowska 1998, Carmelo et al. 1999, Łobacz et al. 2008].

Impedimetric monitoring system employ incubators and a computer equipped with software. The specific characteristic of such system is possibility to incubate food product samples in heating / cooling incubators. This type of analyzer is used for the study of food quality based on determined time of detection (DT) and the obtained linear regression curve. Time of detection means a time after which significant change occurs in the culture medium with electric properties after the influence of the growth of microorganism. Some sort of dependence is observed, the bigger the number of the cells, the shorter is DT. Characteristic traits of this instrument are results of the analysis of the number of microorganisms that are similar to results obtained during the classical plate method [Czajkowska and Witkowska-Gwiazdowska 1998, Zaręba and Tyski 2002].

Impedimetric method in microbiological analysis relies on the phenomenon of a change of electrical conductivity of system, causing change of composition of culture medium, converting weakly dissociated and undissociated macromolecular compounds (polysaccharides, fats, and proteins) to easily dissociating compounds with lower molecular mass, such as organic acids, fatty acids, and amino acids. With accumulation of metabolism products, there is a decrease in resistance of culture medium during the flow of electric current between electrodes submerged in microorganism culture, which is recorded by detector [Kunicka 2000].

Impedimetric method has application for analysis not only dairy, and meat products but for identification of the level of vitamins and antibiotics in food products as well. In addition, a possibility to analysis various products and microorganism species at the same time is a huge advantage of this measurement technique [Czaczyk and Trojanowska 2006].

A key problem of this fast analytical method is lack of developed culture mediums for selective detection of specific bacteria species like *Listeria monocytogenes*.

Therefore, for determination of behavior of this pathogen in diary products, the BHI medium (Biomerieux) with electric properties was enriched by addition of selective factor – Fraser for *Listeria.*

The aim of the work was:

- calibration of Bactometer M64 instrument (Biomerieux) in relation to the plate method for selective determination of *Listeria monocytogenes* cells,
- determination of *Listeria monocytogenes* cells with the use of impedimetric instrument in a feta-type cheese during storage within a range of temperature 3–21^{o}C.

Materials and Methods

Research material

Research material consisted of feta cheese purchased in a retail shop at Olsztyn farm market, before its expiration date.

The characteristic trait of feta cheeses it the type of the raw material used for its production. Traditional brined cheeses are produced of sheep's milk. Currently, they are also produced of cow's milk. The most important stage in the production of these cheeses is their maturation in saline. Typical brined cheese is cubic in shape, submerged in slightly salty whey. The cheese is characterized by white (to creamy) color, clear fragrance, and delicate and salty taste.

Medium

Using the plate method (to calibrate the impedimetric apparatus), medium prepared according to Ottaviani and Agosti (ALOA) was used for surface inoculations. The medium is recommended by PN-EN ISO 11290-2:2000/ A1:2005/ Ap1:2006 /Ap2:2007 standard resulting from regulation (CE) No 2073/2005.

The medium includes selective constituents (nalidixic acid, polymyxin B, cyclohexiimide), which are decomposed by enzymes of *Listeria* sp., and a chromogenic substrate. The colonies are green-blue and are enveloped by a characteristic zone (decomposition of phosphatidylinositol by phospholipase C). Characteristic colonies of *L. monocytogenes* were counted after 48 h of incubation at 37°C.

Calibration of impedimetric monitoring system, Bactometer M64 (Biomerieux)

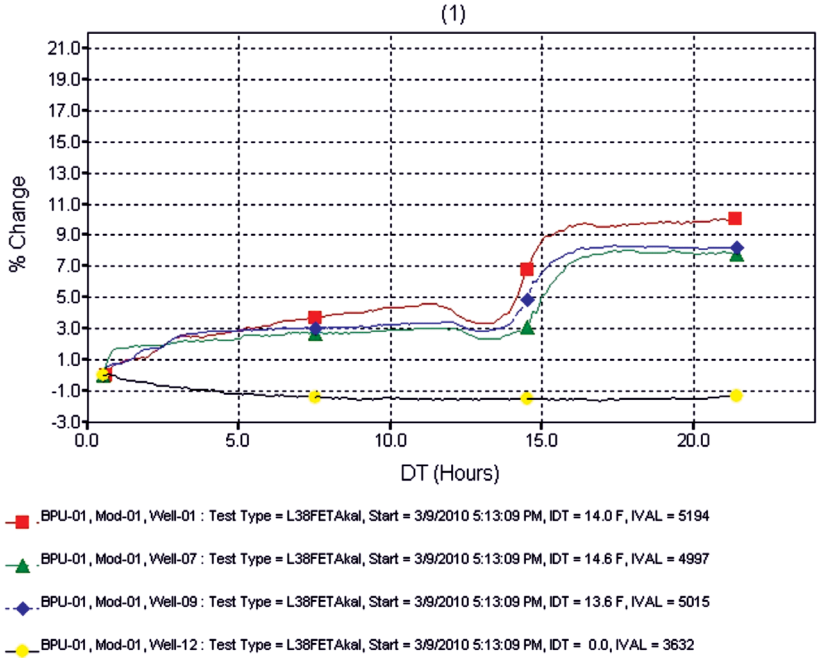
Calibration of the impedimetric apparatus relied on enabling the determination of the number of colonies in 1 g of sample of feta cheese in relation to the classic plate method – with the use of ALOA medium, according to Ottaviani and Agosti.

The amount of 1 cm^3 of the modified BHI medium with electrolytic properties was applied do sterile cells of disposable bactometric module and 1 cm^3 of each following 10 times dilution of feta cheese contaminated with *Listeria monocytogenes* culture. Each cell of the bactometric module was saved in an operating computer program.

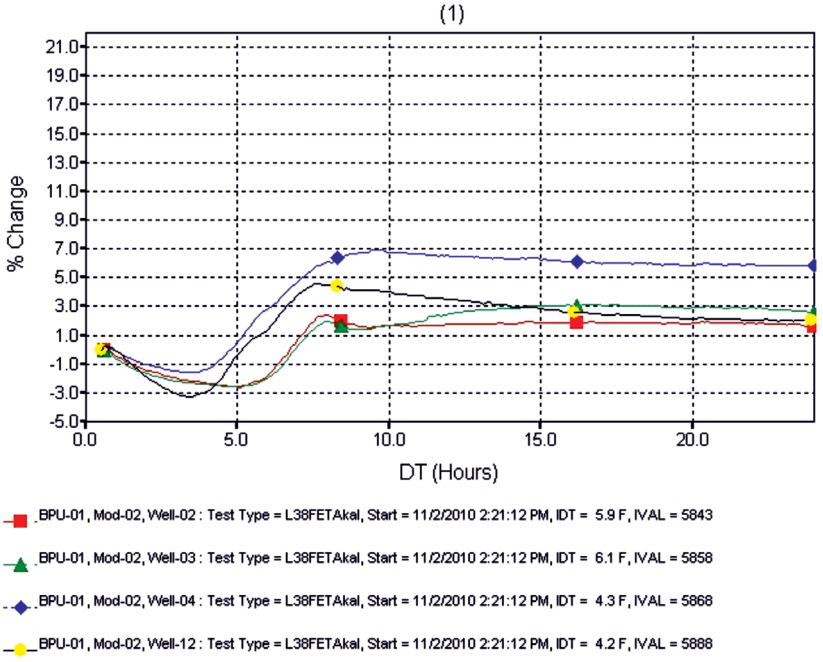
After placing sealed modules in incubator's nests, a stage of 48 h incubation at the temperature optimal for the analyzed microorganism (37°C for *Listeria*) was included.

During the analysis, the computer system plotted a curve resembling the curve of logarithmic growth of microorganisms in relation of the time of detection (DT; [h]) to changes (%) in the medium conductivity.

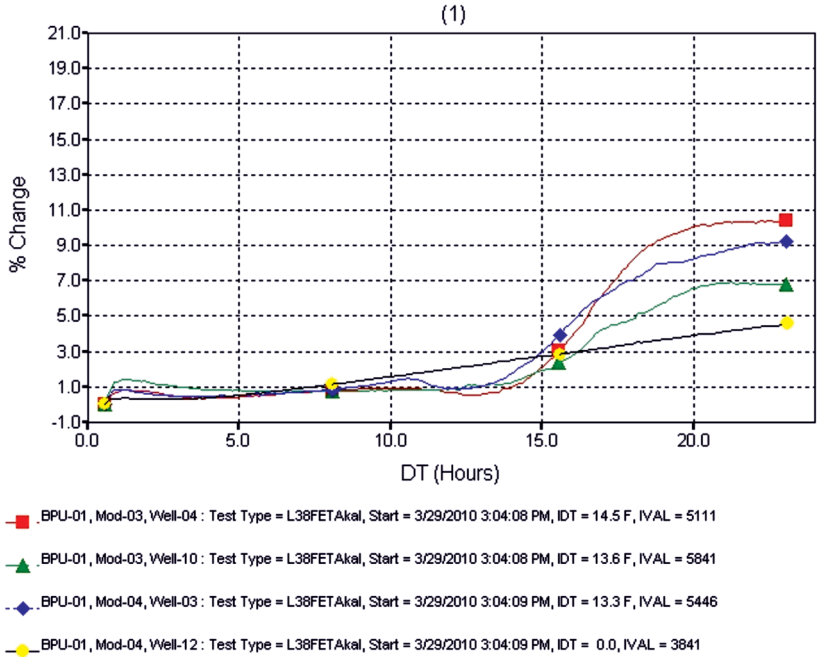
Exemplary plots of the relation of the percentage changes in the medium to DT time [h] from the computer system operating on the Bactometer M64 was presented in Graphs 1–3. GraThe obtained results of analyses in bactometer in the following 10-times dilution series as detection time (DT) were input to a calibration sheet and function of linear regression was set. Type of test and calibration code (designed for bacteria identification) was chosen, and then values read from the plates (classic plate method) were input. The possibility to determine changes in the medium by the bactometer, as log cfu/g (according to the bactometer manual) was established. The operating software plotted a calibration curve (Fig. 2). In addition, control analyzes were performed, that is to the module cells containing modified BHI medium 10 fold dilutions of cheese samples, intentionally not contaminated with *Listeria bacilli* were introduced.



Graph 1. Determination of *Listeria monocytogenes* behavior at the moment of sample contamination with respect for blind sample (lower curve), which did not contain pathogens



Graph 2. Exemplary plot determining *Listeria monocytogenes* behavior after approximately 480 h of storage of cheese samples at 12, 15, and 21°C



Graph 3. Exemplary plot determining *Listeria monocytogenes* behavior after approximately 480 h of storage of cheese samples at 3, 6, and 9 °C with respect to blind sample (lower curve), which did not contain pathogens

Preparation of cheese samples for the determination of *Listeria monocytogenes* cell number growth during storage at 3–21°C

From each production, lot samples of 25 g were weighed and placed in bags used in Stomacher homogenizers (Interscience). They were contaminated with *L. monocytogenes* bacteria culture in the amount to assure cell number at 10^3 CFU g^{-1} (it is the real number in case of potential contamination for example during production).

The culture used for inoculation was obtained as a result of mixing of three reference strains: *Listeria monocytogenes* 0398s (ATCC 7644), *Listeria monocytogenes* 0727s (ATCC 15313), *Listeria monocytogenes* 0232s (ATCC 19112) (Microbiologics). Each of the reference strains was activated as a result of three passages on nutrient broth (incubation at 37°C, for 24 h).

Contaminated cheese samples were stored in precise incubators (Memmert) at the following temperatures: 3, 6, 9, 12, 15, and 21°C. The samples were analyzed in terms of determination of *L. monocytogenes* cell number directly after contamination and in the succeeding days of the experiment. Physiological fluid with peptone (Merck) was used for 10 fold dilutions of the homogenized sample. Inoculations were performed on ALOA selective medium for *Listeria* according to Ottaviani and Agosti (Merck). For each cheese lot before pathogen contamination, assays for *L. monocytogenes* were performed according to PN-EN ISO 11290-1 standard. Therefore, in the first stage, the culture was grown an HalfFraser

medium supplemented with ½ dose of selective supplement at 30°C for 24±3 h, for potentially damaged cell regeneration. Then, the culture was inoculated in Fraser broth. The incubation was conducted for 48 h, at 37°C. HalfFraser and Fraser broths are media containing, beside the nutritional elements (peptons, extracts), also aesculin and lithium chloride. After sterilization, ammonium ferric citrate and selection supplements, that is antibiotic solutions: acriflavin and nalidixic acid (half of the amount in HalfFraser broth) were introduced to the media. Antibiotics and lithium chloride strongly inhibit growth of Gramm-negative and most of Gramm-positive bacteria. Moreover, lithium chloride is a selection factor for the tolerance of higher concentrations of this salt by *Listeria monocytogenes*. *Listeria monocytogenes* strains hydrolyse aesculin to aesculetin and glucose. Aesculetin forms a black complex with Fe(III) originating from the citrate. Culture on this medium, after growth of *Listeria* sp. bacilli, assumes black-grayish or black color. After incubation at 37°C for 48±3, the culture was inoculated on ALOA selective medium for isolation.

In none of the analyzed cheese, lot presence of *L. monocytogenes* was stated. Moreover, during the analyses, pH value of the contaminated cheese was checked using indicator strips (Whatman). The pH value oscillated around 5.1–5.2 during cheese storage at 3–21°C.

Discussion

Modification of BHI (Brain Heart Infusion) medium (own preparation)

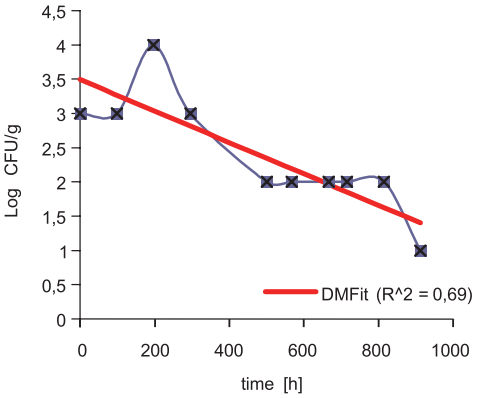
Using proper culturing media and diverse incubation temperature gives the possibility of detection of specific microorganism groups. In order to obtain the results as cell number, the apparatus was calibrated based on plate method for several successive 10-fold dilutions of *Listeria monocytogenes* culture.

To selectively determine the number of *Listeria monocytogenes* cells in the analyzed cheese samples, the BHI bactometric medium was enriched with Fraser supplement used in the determination of *Listeria* presence in PN-EN ISO 11290-2:2000. The supplement contains ammonium ferric citrate responsible for *Listeria* growth and selective supplement (inhibition of accompanying microflora during *Listeria* growth).

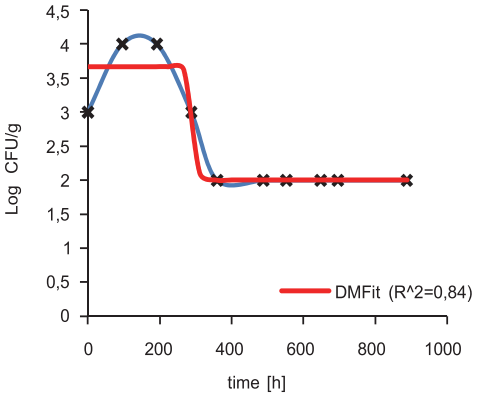
Determination of the behavior of *Listeria monocytogenes* strains in feta cheese during storage at 3–21°C using a calibrated Bactometer M64 system

During the measurement days, the contaminated cheese sample was submitted to homogenization in Stomacher type device and incubated in bactometric modules containing modified, selective medium. Results of the measurement were in form of detection time DT, and interpreted by the impedimetric system operating software in decimal form (log CFU g^{-1}) (Fig. 2) with respect to the 10-fold dilutions of homogenized samples. The system used the parameters of analysis for the determination of linear regression equation (Fig. 3).

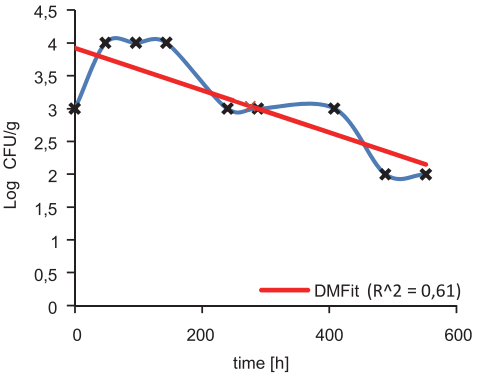
The results (presented in Graphs 4–9) obtained during the analysis of *Listeria monocytogenes* cell number growth during feta cheese storage at 3-21°C were fitted to Baranyi and Roberts model using Excel application (MS Office) – DMFit (DM: Dynamic Modelling) [Baranyi 1994].



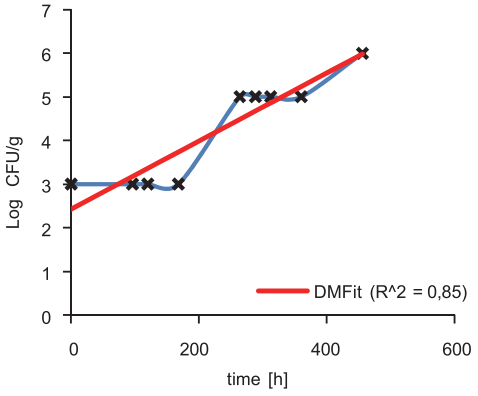
Graph 4. *Listeria* in feta cheese (temperature of 3°C)



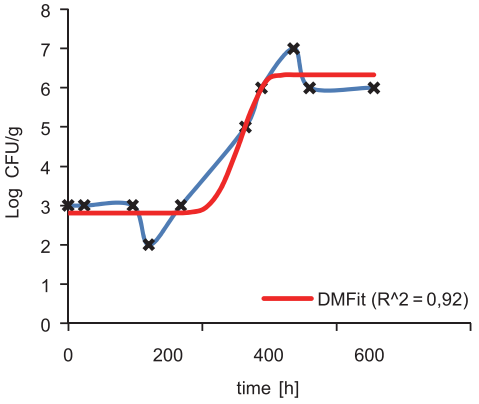
Graph 5. *Listeria* in feta cheese (temperature of 6°C)



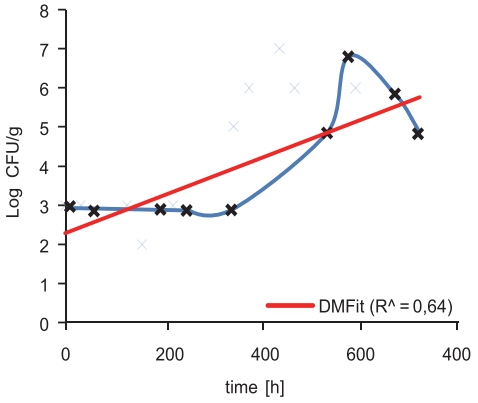
Graph 6. *Listeria* in feta cheese (temperature of 9°C)



Graph 7. *Listeria* in feta cheese (temperature of 12°C)



Graph 8. *Listeria* in feta cheese (temperature of 15°C)



Graph 9. *Listeria* in feta cheese (temperature of 21°C)

DMFit application was constructed based on Baranyi model [1] [Baranyi 2002]. It is a dynamic model (primary) describing microorganism growth, used in predictive microbiology.

Lag phase is determined as the time required for q substrate synthesis, which is crucial for microorganism growth. When a bacterial cell accommodates to a new environment, its number increases exponentially until limitations from medium appear:

$$\frac{dx}{dt} = \frac{q(t)}{q(t)+1}\mu_{\max}\left(1-\left(\frac{x(t)}{x_{\max}}\right)^{m}\right)x(t) \qquad [1]$$

where:
x – number of cells in time (t),
x_{max} – maximum cell density,
m – parameter characterizing change of the growth curve to stationary phase,
q(t) – concentration of crucial substrate, which changes in time (t):

$$\frac{dq}{dt} = \mu_{\max} q(t)$$

[McKellar and Lu 2004].

Slight (by about 1 log CFU g^{-1}) growth in *Listeria* cells number from the initial level of log 3 cfu/g was observed during storage at 3–9°C (Graphs 4–6), but after about 200 h of storage, a dropdown in *Listeria monocytogenes* cells number occurred. Graphs 7–9 (cheese sample storage temperature of 12–21°C) present increase in *Listeria monocytogenes* cell number. The highest index of *Listeria* cell number growth rate μ = 0.049 [log10 CFU h^{-1}] was calculated in DMFit application for sample storage at 15°C. Similar results were obtained by Back et al. [1993] in the research on soft cheese containing 2.5% NaCl (Fig. 1), where μ = 0.014. The analyses were performed at storage temperature of 4–15°C.

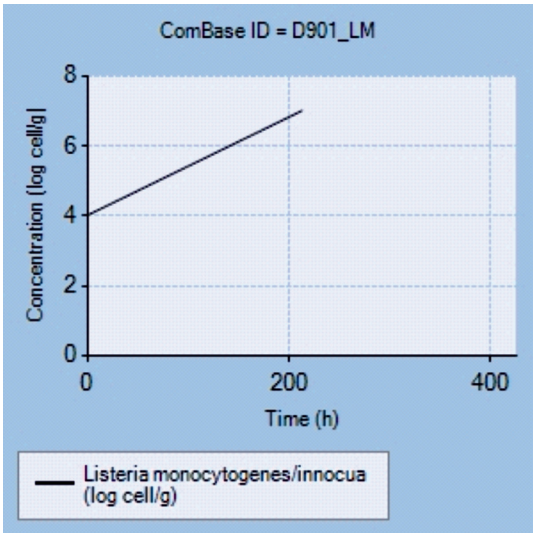


Fig. 1. Exemplary growth of *Listeria monocytogenes* in soft cheese at 15°C (pH = 5.2, NaCl = 2.5%) (source: database www.combase.cc)

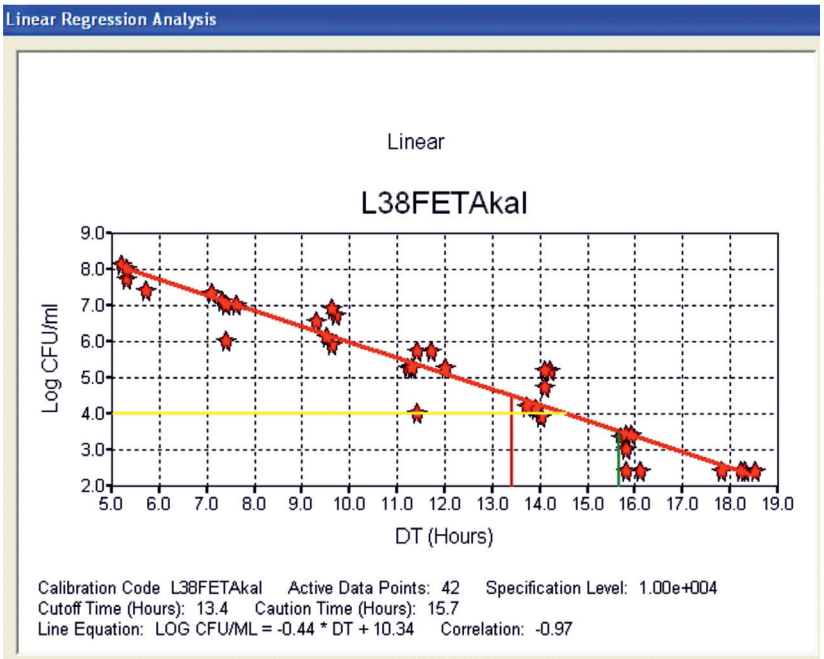


Fig. 2. Calibration curve obtained in impedimetric system Bactometer M64 for determination *Listeria monocytogenes* cell number in feta cheese

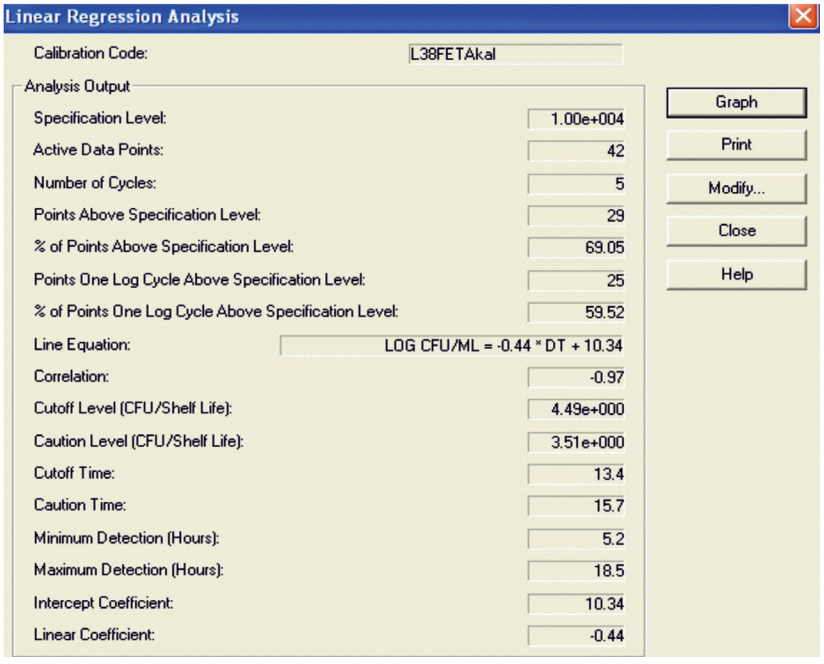


Fig. 3. Parameters determining among others the linear regression equation obtained in Bactometer M64 operating software

Summary

Based on the obtained results, there exist possibilities of microbiological media preparation, which facilitate quantitative determination of the amount of various species of pathogenic bacteria in different food products (including dairy products).

Quantitative determination of pathogenic bacteria cells is more and more often required by the regulations (CE 2073/2004 with later changes in the CE 1441/2007 regulation) and is a basis for quantitative analysis of the microbiological risk during food production and distribution.

Alternative methods quantitatively determining the microbiological risk allow for flexible supporting of systems ensuring the safety of production (for example HACCP).

The method using the impedance allows for quicker than the traditional plate method quantitative determination of microbiological threats at various stages of food production, during recipe changes, process parameters, and during storage, distribution, and establishing the expiration date.

Acknowledgements

This work was financially supported by the Ministry of Science and High Education. Project N N312 296935.

References

Back J.P., Langford S.A., Kroll R.G., 1993. Growth of *Listeria monocytogenes* in Camembert and other soft cheeses at refrigeration temperatures. Journal of Dairy Research., 60, 421–429.

Baranyi J., 2002. Stochastic modelling of bacterial lag phase. 2002. Int. J. Food Microbiol., 73, 203–206.

Baranyi J., Roberts T.A., 1994. A dynamic approach to predicting bacterial growth in food. Int. J. Food Microbiol., 23, 277–294.

Carmelo J.F, Madrid R.E, Olivera J.M., Rotger V.L., 1999. Impedance microbiology: quantification of bacterial content in milk by means of capacitance growth curves, J. Microbiol Meth., 35, 37–42.

Czaczyk K., Trojanowska K., 2006. Zastosowanie szybkich metod oznaczania i identyfikacji drobnoustrojów. Przem. Spoż., 2, 17–20.

Czajkowska D., Witkowska-Gwiazdowska A., 1998. Metoda impedymetryczna w wykrywaniu drożdży bakterii fermentacji mlekowej w sokach z owoców cytrusowych. Przem. Spoż., 12, 14–15.

Kijowski J., Cegielska- Radziejewska R., 2008. Kontrola zagrożeń żywności auditowanym i certyfikowanym systemem ISO 22000/ HACCP. Wydawnictwo Poznań, Poznań, 32, 46–70.

Kowalik J., Łobacz A., Tarczyńska S., Ziajka S., 2009. Zastosowanie mikrobiologicznych modeli prognostycznych w produkcji bezpiecznej żywności. Medycyna Wet., 65(6), 381–384.

Kunicka A., 2000. Wykorzystanie metody impedymetrycznej w analizie mikrobiologicznej, Laboratoria Aparatura Badania, 5(5), 18–21.

Łobacz A., Kowalik J., Ziajka S., 2008. Wykorzystanie zjawiska impedancji w mikrobiologii i higienie żywności. Medycyna Wet., 8 (64), 966–968.

Maćkiw E., Szponar L., 2003. Podstawy analizy ryzyka bezpieczeństwa żywności w aspekcie mikrobiologicznym. Bromat. Chem. Toksykol. XXXVI, 3, 201–208.

Mafart P., 2005. Food engineering and predictive microbiology: on the necessity to combine biological and physical kinetics. Int J Food Microbiol., 100, 239–251.
McKellar R.C., Lu X.W., 2004. Modeling microbial responses in foods, CRC Press LLC, Florida.
Nowak A., 2007. Interpretacja Rozporządzenia Komisji (WE) NR 2073/2005 w sprawie kryteriów mikrobiologicznych dotyczących środków spożywczych. Chłodnictwo, 3, 60–62.
Rozporządzenie (WE) nr 178/2002 Parlamentu Europejskiego i Rady z dnia 28 stycznia 2002 r. ustanawiające ogólne zasady i wymagania prawa żywnościowego, powołujące Europejski Urząd ds. Bezpieczeństwa Żywności oraz ustanawiające procedury w zakresie bezpieczeństwa żywności
Ryser E.T., 2002. *Listeria monocytogenes*, [in:] Encyclopedia of Dairy Science, red. Rogiński H., Academic Press, London, 1650–1655.
Windyga B., Ścieżyńska H., 2010. Jakość mikrobiologiczna żywności w Polsce. Przem. Spoż., 10, 8–10.
Zaręba T., Tyski S., 2002. Zastosowanie metody impedymetrii w badaniu skuteczności działania środków konserwujących leki. Med. Dośw. Mikrobiol., 54, 89–90.

6

APPLICATIONS OF ENZYMATIC METHODS AND LIQUID CHROMATOGRAPHY (HPLC) FOR DETERMINATION OF CARNITINE CONTENT IN MILK AND MEAT

Introduction

In recent years the amino acid derivative – L-carnitine has gained increased interest as a naturally occurring compound of animal origin that has a beneficial effect on humans health. Carnitine (β-hydroxy-γ-trimethylaminobutyrate) is a low molecular weight quaternary amine present in almost all animal species, as well as in several microorganisms and in some higher plants [Alesci et al. 2003].

The primary function of L-carnitine is to permit the entry of esterified fatty acids (the source of ATP synthesis) into the mitochondrial matrix, where β-oxidation occurs. Short- and medium-chain fatty acids are transported into the mitochondrial matrix without any carnitine assistance in the process. Long-chain fatty acid acyl groups are transported exclusively as carnitine esters by a carnitine carries called the translocase, which constitutes as a transmembraneous protein in the inner mitochondrial membrane. Despite this seemingly simple role, this function is significant for fatty acid β-oxidation as well as maintenance of the acyl-coenzyme A (acyl-CoA) [Seim et al. 1980]. The ability of L-carnitine to accept/and donate acyl-groups from/and to the corresponding acyl-coenzyme A thioester is integral to the physiological functions of the compound. In addition long-chain fatty acids constitute a basic substrate for oxidative energy metabolism in the myocardium (the muscular tissue of the heart) [Evans et al. 2000]. Thereby cardiovascular system function is strictly related to carnitine depended metabolism.

In mammals, L-carnitine is synthesized mainly in the liver, kidneys and in some species in the testes and the brain from essential amino acids lysine and methionine as ultimate precursors [Vaz and Wanders 2002]. The total body pool comprises, besides L-carnitine, various carnitine esters and more than 90 % of the pool resides within skeletal and myocardial muscle, with the remainder being present in the liver, kidneys and other tissues [Lamhonwah et al. 2009].

The carnitine homeostasis is maintained by a modest rate of inner carnitine synthesis, dietary intake and efficient management of carnitine by the kidney. Thus the daily requirement for L-carnitine (ca 15 mg) is supplied to the body through endogenous biosynthesis

and exogenous sources [Ramsay et al. 2001]. Skeletal muscles as the main reservoir are characterized by L-carnitine concentration at least 50 to 200 times higher than in blood plasma [Evans and Fornasini 2003], that is why meat is considered as the best choice for adding carnitine into the diet. According to literatures data the primary source of L-carnitine is red meat, particularly lamb and beef, while a lower carnitine content is found in fish, pork and poultry. The dairy products such as whole milk or cottage cheese, constitute the good carnitine source as well. In plant origin products L-carnitine occurs just in trace quantities or does not occur at all [Knuttel-Gustavsen and Harmeyer 2007, Li et al. 1990]. Most individuals consume carnitine in excess of apparent maintenance demands, however the knowledge about L-carnitine concentrations in different biological materials is helpful when humans are suffering from carnitine deficiency and an exogenous supplementation is needed [Czeczot and Ścibol 2005].

The hallmark of carnitine deficiency is low concentrations of carnitine in plasma and targeted tissues, with accumulation of lipid deposits and renal leakage of carnitine. This specific disease is characterized by progressive cardiomyopathy and skeletal muscle dysfunction. Myopathic (muscle) carnitine deficiency symptoms include muscle weakness, enlargement of cardiac muscle and development of ischemic heart disease [Rinaldo et al. 2003, Shoji et al. 1998].

With respect to the increasing meaning of consuming full value nutriment the functional food is a subject of a growing interest among consumers all over the world. The aim of the present paper was estimation of genetic factor influence on carnitine content. Accordingly study on comparison of different analytical methods used for determination carnitine levels in animal derived products (milk and meat) was conducted.

Material and Methods

Raw milk and fresh meat samples for research were obtained from facilities in cooperation with Institute of Animal Breeding, University of Environmental and Life Sciences in Wroclaw.

Milk samples for research have been collected during May, from 3 different ruminant animal species: cows (HF cattle red-white type, Malerzowice Małe farm), goats (Polish White Ennobled race, "Kozio-lek" farm) and sheeps (Polish Mountain race, "Puchaczówka" mountain station).

All of the animals were in 2–3 stage of lactation, in the first half of particular stage.

The nutrition was based on traditional fodders (applied on farms the milk samples were collected from) which were proportioned according to mandatory nutritional standards. In the case of cows it was maize and grass silage, spent grain, beet pulp and concentrate fodders (rapeseed cake, maize, barley); in the case of sheep pasture silage, meadow hay and wheat bran; in the case of goats pasture silage, meadow hay, concentrate fodder and brewing industry wastes.

The samples of meat for the analyses originated from longest deep back muscle (*musculus longissimus dorsi*) from 15 animals of each species: 6 month old young beef cattle (young bulls) of HF breed of black-white variety ("Agrominor" Mokrzeszów), 3 months old lambs

(rams) of Polish Merino breed ("Agrominor" Mokrzeszów) and 3 months old goats (billy goats) Polish White Ennobled race ("Kozio-lek" farm).

Traditional fodders used in these species feeding were applied in the study, and were proportioned according to mandatory nutritional standards. Young bulls were fed with TMR, meadow hay, concentrate fodder and extracted rapeseed meal; rams with concentrate fodder (cereal fodders, extracted rapeseed meal, dried green fodder) and meadow hay, goats with pasture green fodder, meadow hay and concentrate fodder.

The samples from all animals of each species were collected twice then prepared for determination of total (free and esterified) and free L-carnitine.

Methods for the detection and quantification of carnitine have used a variety of strategies. In order to compare utility of high-performance liquid chromatography method and spectroscopic methods for carnitine quantification in biological samples, two procedures (spectrophotometric and spectrofluorometric) based on enzymatic reactions were conducted. Other analytical method which use HPLC separation followed by UV detection of derivatized carnitines [Kempen and Odle 1992, Minkler et al. 2005] has been applied.

Part I. Spectroscopic methods

Milk samples preparation methods for direct estimation of total carnitine, free L-carnitine and acylcarnitines content have been modified. Final procedure has been established and summarized in Figure1.

Total L-carnitine concentration was estimated using alkaline hydrolysis (2 M KOH, 60°C, 1hr). After cooling, the extract was neutralized to pH 6.5 ± 0.5 with perchloric acid (70% w/v), transferred to a volumetric flask (100 ml) and made to volume with distilled water. The precipitate was removed by filtration, discarding the first portion and collecting 20 ml. Approximately 5 ml was clarified by passage through a 0.45 μm membrane and kept under refrigeration (4°C) until ready for assay. Free L-carnitine form was determined by deproteination applied as a first step. Perchloric acid (0.25 M, 40 ml) was added, mixed gently and allowed to stand for 60 min. The sample was filtered into a flask and 20 ml collected after discarding the first filtrate. The extract was adjusted to pH 6.5 ± 0.5 with potassium hydroxide (1.0 M). Thorough stirring was necessary to avoid localized alkaline conditions and thereby minimize acylcarnitine hydrolysis. The solution was transferred quantitatively to a volumetric flask (50 ml), and made to volume with water. Then, final clarification depending on the filtration through a 0.45 μm membrane was followed. An aliquot was transferred directly to a vial (5 ml). Carnitine extracts are stable for several weeks at 4°C they may be held until required for assay.

Meat samples (20 g) required previous homogenization in 10 ml of cold deionized water and 10 ml of 0.5 M $HClO_4$ with mechanical laboratory homogenizer. Then homogenized tissue was centrifuged with 5 ml of 1 M KOH in methanol for 10 min at 13 000 × g. The supernatant was transferred into another flask, and neutralized with K_2CO_3 to pH 7 ± 0.5. After neutralization the prepared solution was processed accordingly to the procedures presented in Figure1 in respect to which of carnitine form was chosen for determination. Milk and meat samples after proper preparation were analyzed with two enzymatic methods – spectrophotometric and spectrofluorometric.

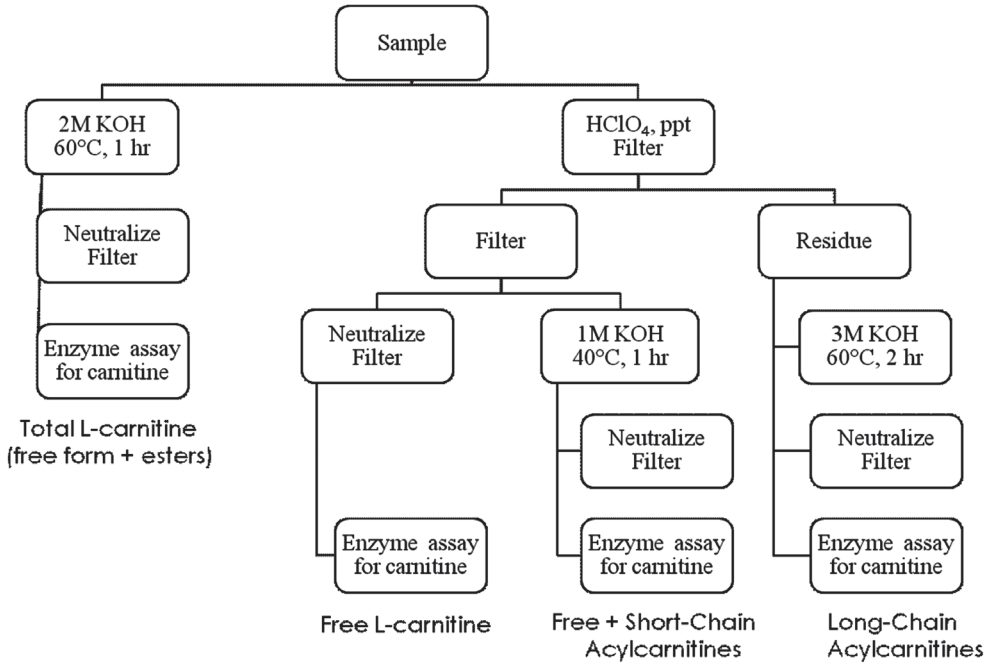


Fig. 1. States in preparation of a milk samples for analysis

Spectrophotometric method (Method I)

The enzymatic determination protocol has been previously described [Indyk and Woollard 1997] as a reliable analytical method for the routine determination of total an free carnitine form. Method I was based on reversible enzymatic acetylation reaction of free L-carnitine catalyzed by carnitine acetyltransferase (CAT, E.C. 2.3.1.7). The enzyme isolated from pigeon breast muscle (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) enabled acetyl group transport from acetyl-CoA to L-carnitine which resulted in release of free CoA molecule. The spectrophotometric assay is based upon the premise that carnitine present will permit the liberation of an equimolar amount of free CoA under conditions in which the initial concentrations of acetyl-CoA, carnitine acetyltransferase, and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) are held constant. Free CoA interact with DTNB: the combination of DTNB (Ellman's reagent) with sulfhydryl groups forms the thiophenolate ion which absorbs at 412 nm. The thiophenolate ion has been forming in proportional amount to L-carnitine content in sample. The optimal pH for DTNB ranges 7.5–10.4 while for CAT enzyme 7.0-8.0, thus both reaction were managed in the same volume, in phosphate buffer of pH 7.5. After 15 min of incubation absorption at 412 nm was measured.

Spectrofluorometric method (Method II)

Furthermore, modified spectrofluorometric method using combined enzymatic systems was utilized. Method II depended on two enzymatic reactions: L-carnitine interactions with

acetyl-CoA resulting in formation of free CoA molecules which reacted with 2-oxoglutarate in presence of nicotinamide adenine dinucleotide (NAD^+). First enzymatic reaction, described in previous (method I) section, was catalyzed by CAT enzyme and in second reaction enzymatic complex isolated from porcine heart muscle was utilized. The oxoglutarate dehydrogenase (OGDH, E.C. 1.2.4.2, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) was the applied reagent. OGDH which required thiamine diphosphate serving as a cofactor catalyzed oxidative decarboxylation reaction, where succinyl-CoA and reduced form of nicotinamide adenine dinucleotide (NADH) were obtained as a products. Admitting of the optimal pH for OGDH complex activity (7.2–8.0) and for CAT enzyme (7.5–8.0) analysis was conducted in environment of phosphate buffer (pH 7.5). Change in absorbancy was observed and measured, what was certifying NADH content change and thereby defining L-carnitine concentration in sample. Both reactions, which were usually completed within ca 20 min after addition of the last component, was allowed to proceed until no further absorbancy change occurred.

For each of described methods (method I and II) individual standard curve was obtained by determination of absorbancy changes at 412 nm and 340 nm after known amounts of carnitine (in range 10–400 μmol/l) were added to the analytical systems. Particular absorbancy values (returned by apparatus, Pharmacia LKB, Biochrom 4060) constituted a foundation to free and total L-carnitine form concentration in relation to relevant standard curve.

Part II. HPLC method

The presented method provides a powerful tool to analyze L-carnitine and carnitine esters concentration. Because it employs standard chromatographic column and commercially available reagents, this method is easy to implement in laboratory. The HPLC/UV system consisted of an Perkin-Elmer apparatus, quaternary LC pomp 200 series and diode array detector. The analytical column was a 100A, 50×4.6 mm i.d. C18 column purchased from Kinetex Group with hexanoylo-D,L-carnitine served as a internal standard. Commercially available (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) L-carnitine chloride, acetyl-L-carnitine chloride and O-acetyl-L-carnitine chloride was used to obtain standard elution profile.

Milk and meat samples preparation was achieved after modification of Minkler and Janssens combined procedures [Janssens et al. 1999, Minkler et al. 1995]. Tissues were homogenized in cold deionized water. To glass centrifuge tube 0.05 ml of saturated potassium phosphate monobasic and 0.05 ml of hexanoyl-D,L-carnitine chloride were added and vortex-mixed for 5 sec. Next 50 μl of standard or tissue homogenate was added. The tube was capped, mixed and centrifuged (5 min, 13 000 × g). The entire supernatant from extracted tissue was collected and evaporated to dryness. Following step required molecules derivatization to gained phenacylcarnitine esters which have maximum UV absorption at 245 nm. Acylcarnitines were derivatized using commercially available 2,4'-dibromoacetophenon which reacts with carboxyl group of carnitine forming phenacyl ester [Durst et al. 1975]. The dry fraction was reconstituted in 20 μl of water, 0.1 ml of derivatization reagent (0.1 M 2,4'-dibromacetophenon and 0.005 M 18-Crown-6 ether in acetonitrile) and 0.9 ml of acetonitrile. This composition was heated at 80°C under stirring for 90 min. The reaction mixture was evaporated to dryness, and phenacylcarnitine esters were reconstituted in 500 μl of

a mixture of acetonitrile/water (25/75, v/v). Up to 50 µl of this mixture were injected into HPLC system.

Three chromatographic eluents were employed to formulate the gradient described; eluent A, acetonitrile/water (70/30, v/v); eluent B, water (100%), eluent C, acetonitril/water/triethylamine/phosphoric acid (acetonitrile-TEAP, 80/20/0.8/0.64, v/v/v/v). A constant flow--rate of 1.6 ml/min was utilized. The eluent A was used to equilibrate the column and was maintained until 1 min after an injection was made. Then, the switch valve was activated on pump A, causing a flow of 100% water (eluent B). The acetonitrile remaining in the system was sufficient to eluate all excess derivatization agent. Eluent B was subsequently maintained at 100% for 5 min. At 6 min after injection, a gradient was initiated, delivered through pump B, with the acetonitrile-TEAP eluent (eluent C) initially at 15%. This was increased linearly over 25-min time period to 100% (replacing eluent B). Then, 36 min after injection, acetonitrile-TEAP was switched to 100% eluent A (via pump A). The system was re-equilibrated for about 20 min; injections could thus be made at ca 60-min intervals.

Derivatives of the free L-carnitine and also L-carnitine esters were distinguished and retention times were determined in order to identify peaks in chromatograms obtained from biological samples.

Results and Discussion

Results of total and free L-carnitine determination in raw milk and fresh meat have been presented in Tables 1, 2.

Both spectroscopic (I method – spectrophotometric, II method – spectrofluorometric) and chromatography HPLC methods returned similar results which confirmed specific differences in total and free L-carnitine content in biological samples of milk in respect to particular genotype (Tab. 1).

The highest level of both carnitine forms was stated in sheep milk, medium in cow, and lowest was found in goat milk.

In sheep milk total carnitine concentration, depending on used method, is about 36.7 (spectrophotometric method) and respectively 39.7% (chromatography method) ($P \leq 0,05$) higher than in cow milk, and when compared with goat accordingly 88.1 (chromatography method) and 100% (spectrofluorometric method) ($P \leq 0,01$) higher.

In case of the free L-carnitine form determined with spectrophotometric method the distinction between sheep and cow milk and also sheep and goat amounts respectively to 73.2 and 243.2% ($P \leq 0,01$), in turn between cow and goat 98.2% ($P \leq 0,01$). Whereas approximate results were obtained by spectrofluorometric method: distinction between sheep and cow milk 60.4% ($P \leq 0,01$), sheep and goat 255.7% ($P \leq 0,01$) or cow and goat 121.7% ($P \leq 0,01$). Similarly to spectroscopic methods, interspecies diversities of free L-carnitine level in milk were confirmed by chromatographic analyses with HPLC application. In sheep milk free L--carnitine level was about 50.8 ($P \leq 0,01$) higher than in cow milk, and about 201.6% ($P \leq 0,01$) higher than in goat milk. Statistically important differences were also established between cow and goat milk. Cow milk contained about 100 % more of total L-carnitine than goat milk.

After spectroscopic and HPLC analysis the research results shed also a light on free:total L-carnitine ratio set in different milks (calculation not shown). The most profitable

Table 1

Content of total and free L-carnitine in milk of different animal species determined with spectrophotometric (I), spectrofluorometric (II) and HPLC methods

Milk descent	Statistical symbol	Total L-carnitine [mg/100 ml]			Free L-carnitine [mg/100 ml]		
		method I	method II	HPLC	method I	method II	HPLC
Cow	**X** ± Sd (min – max)	**8.31**^{a} ± 0.32 (7.74–8.86)	**8.42**^{a} ± 0.28 (8.06–8.60)	**8.24**^{a} ± 0.31 (7.91–8.60)	**2.20**^{A} ± 0.13 (2.06–2.58)	**2.35**^{A} ± 0.15 (1.96–2.62)	**2.46**^{A} ± 0.14 (2.16–2.69)
Goat	**X** ± Sd (min – max)	**6.02**^{A} ± 0,46 (5.06–6.52)	**5.88**^{Ab} ± 0.76 (4.60–7.02)	**6.12**^{Ab} ± 0.80 (5.11–7.46)	**1.11**^{B} ± 0.04 (0.97–1.18)	**1.06**^{B} ± 0.05 (1.02–1.18)	**1.23**^{B} ± 0.06 (1.06–1.32)
Sheep	**X** ± Sd (min – max)	**11.36**^{Ab} ± 0.59 (10.02–12.32)	**11.76**^{Bb} ± 0.61 (10.24–12.10)	**11.51**^{Bb} ± 0.46 (10.46–12.72)	**3.81**^{C} ± 0.16 (3.42–4.02)	**3.77**^{C} ± 0.17 (3,.50–3.92)	**3.71**^{C} ± 0.14 (3,.46–4.07)

^{a,b} – statistical significance on the level p≤0,05

^{A,B} – statistical significance on the level p≤0,01

free:total L-carnitine proportion was characteristic to sheep milk and amounts 0.32, while in cow milk 0.28 and goat 0.19.

The analyses confirmed data published in accessible literature [Patkowska-Sokoła et al. 2003, Indyk et al. 1997] and also in paper concerning radio-enzymatic assay of L-carnitine based on radioactive labelling of free L-carnitine form in the sample [Knüttel-Gustavsen and Harmeyer 2007].

Despite its relevance to human health, there is limited information about the carnitine content and distribution in foods. Information about bovine milk and derived dairy products is incomplete, although some data have been reported [Bosi and Refrigeri 1983, Indyk and Woollard 1995].

Some information concerning radioisotopic determination of L-carnitine content in meat have also been published [Demarquoy et al. 2004], furthermore they lent support to results of spectroscopic and HPLC methods analysis of meat samples presented in Table 2.

In meat, likewise in milk, interspecies diversities of free and total L-carnitine level in meat were found. The highest concentration of these was found in lamb, consecutively in beef and goat meat (Tab. 2).

Total L-carnitine content in lamb was higher about 11.7% ($P \leq 0,05$) (spectrophotometric method), 11.5% (spectrofluorometric method) and 12.2% ($P \leq 0,05$) (HPLC) when compared to goat meat. Lamb was also characterized by 7% (spectrophotometric method), 6.3% (spectrofluorometric method) and 6.7% (HPLC) higher level of total L-carnitine than beef. Differences was therefore also established between beef and goat meat. Beef contained about 5.8% (enzymatic method I), 4.9% (enzymatic method II) and 5.2% (chromatography method) more of total L-carnitine than goat meat.

In case of the free L-carnitine form determined with spectrophotometric method the distinction between lamb and beef meat and also lamb and goat amounts respectively 11.5% and 22.4% ($P \leq 0,05$), in turn between beef and goat 9.8%. Approximate results were obtained by spectrofluorometric method: distinction between lamb and beef 15.3%, lamb and goat 25.1% ($P \leq 0,05$) or beef and goat 8.6%. Similarly to spectroscopic methods, interspecies diversities of free L-carnitine level in meat were confirmed by HPLC analytical method. In lamb free L-carnitine was about 14.5% higher than in beef and about 22.30% ($P \leq 0,05$) higher than in goat meat. It was also found that beef contained 8.2% more of free L-carnitine concentration than goat.

The most profitable free:total L-carnitine proportion was characteristic to lamb meat and amounts 0.82, while in beef 0.77 and goat 0.75.

To assess the presence of L-carnitine in meat products, free L-carnitine amount was estimated in various eaten meat products of domestic ruminant species. As a general rule, gained data confirmed the presence of L-carnitine, in relatively high concentration, in basically each meat product analyzed. These included beef, goat and lamb, among which, lamb muscle appeared as the best source for L-carnitine.

Summary and Conclusions

The object of this paper was to describe a methods to analyze carnitine esters using commonly available equipment and chemicals. The colourimetric detection mode places some constraints on the sample preparation strategy selected for free and total carnitine estimation. Two

Table 2

Content of total and free L-carnitine in meat of different animal species determined with spectrophotometric (I), spectrofluorometric (II) and HPLC methods

Meat descent	Statistical symbol	Total L-carnitine [mg/100 mg]			Free L-carnitine [mg/100 mg		
		method I	method II	HPLC	method I	method II	HPLC
Goat	**X** ± Sd (min – max)	**96.42** ± 0.47 (95.26–98.96)	**97.11** ± 0.76 (95.33–99.17)	**97.20** ± 0.52 (95.42–99.36)	**72.86**[a] ± 0.56 (70.13–74.11)	**71.96** ± 0.63 (70.46–73.29)	**73.18**[a] ± 0.48 (72.76–74.87)
Beef	**X** ± Sd (min – max)	**102.06** ± 0,66 (100.11–104.06)	**101.85** ± 0.54 (100.23–103.94)	**102.23** ± 0.62 (100.24–104.46)	**80.02** ± 0.76 (78.36–82.11)	**78.11** ± 0.62 (76.87–81.16)	**79.18** ± 0.72 (77.21–81.86)
Lamb	**X** ± Sd (min – max)	**109.21** ± 0.56 (107.02–111.89)	**108.32** ± 0.64 (106.97–110.89)	**109.09** ± 0.79 (107.16–111.32)	**89.21**[b] ± 0.51 (87.08–98.11)	**90.04**[a] ± 0.59 (87.96–92.79)	**89.50**[b] ± 0.63 (87.92–91.82)

[a,b] – statistical significance on the level $P \leq 0,05$

protocols of sample preparation for spectroscopic methods were evaluated, involving either hydrolysis of the intact sample, or removal of fat and protein prior to alkaline hydrolysis. Both of the protocols were efficient and allowed for carnitine determination with high yield of recovery.

Three presently described analytical methods for determination of free and acylated forms of carnitine were utilized to generate information concerning the levels in milks and meats of differing origin.

Standard colourimetric method (method I) following a previous biological sample preparation, with use of the coupled carnitine acetyl transferase-Ellman reaction tandem allows for carnitine and its esters measurement and has been established for inexpensive and easier to apply in comparison to method II. Even when compared to advanced HPLC method the analysis time of method I was shorter, and the prices of applied reagents were also lower. However, HPLC method can be characterized by better detection limit (ca 20 pmol) but requires expensive chemicals, apparatus and well qualified and skilled personnel as well.

All of the applied methods have given comparable and reproducible results which confirmed that the content of quantified carnitine forms was significantly diversified in milk and meat samples from the three domestic ruminant species. The highest concentration of L-carnitine in tested samples was found in a sheep milk (11.54 mg of total carnitine/100 ml; 3.76 mg of free L-carnitine/100 ml) and in a lamb (108.9 mg of total carnitine/100 mg; 89.6 mg free L-carnitine/100 mg). In general, it has been proved that muscles constitute a good source of L-carnitine, moreover dairy product are able to provide meaningful quantities of the important isomer in human diet as well.

With respect to carnitine deficit and the highly dangerous risk for the cardiovascular system caused by it, consuming full value nutriment is essential. Understanding what determines the L-carnitine level in living organisms could allow for enriching food in this important compound [Pękala et al. 2011]. Such information will be increasingly required in view of constructing balanced feeding for those with L-carnitine extended demand.

Acknowledgements

The study was conduced within the framework of The Polish Ministry of Science and Higher Education Grant no N N311 019537 "L-carnitine biologically active stereoisomer with prohealth properties – study on different factors influence on L-carnitine levels in milk and meat of ruminant animals and also research on possibilities in increasing L-carnitine concentration in these animal products". The work was partially cofinanced by European Union within European Social Found by Fellowship for doctorants and postdocs of Wroclaw University of Technology "Didactic-research development of young academic management" No MK/SN/204/IV/2011/U.

References

Alesci S,. De Martino M.U., Mirani M., Benvenga S., Trimarchi F., Kino T., Chrousos G.P., 2003. L-Carnitine: a nutritional modulator of glucocorticoid receptor functions. The FASEB Journal, 17, 1553–1555.

Bosi G., Refrigeri M.S., 1983. Determinazione gas-chromatografica della carnitina nel latte. Rassegna Chim., 35, 421–426.
Czeczot H., Ścibol D., 2005. Role of L-carnitine in metabolism, nutrition and therapy. Postępy Hig. Med. Dosw. Epub, 59, 9–19.
Demarquoy J., Georges B., Rigault C., Royer M.C., Clairet A., Maud Soty M., Lekounoungou S., Borgne F., 2004. Radioisotopic determination of L-carnitine content in foods commonly eaten in Western countries. Food Chem., 86, 137–142.
Durst H.D., Milano M., Kikta E.J.Jr., Connely S.A., Grushka E., 1975. Phenacyl esters of fatty acids via crown ether catalysts for enhanced ultraviolet detection in liquid chromatography. Anal. Chem., 47, 1797–1801.
Evans A.M., Faull R., Fornasini G., Lemanowicz E., Longo A., Pace S., Nation R., 2000. Pharmacokinetics of L-carnitine in patients with end-stage renal disease undergoing long-term hemodialysis. Clin. Pharmacol. Ther., 68(3), 238–249.
Evans A.M., Fornasini G., 2003. Pharmacokinetics of L-carnitine. Clin. Pharmacokinet., 42, 941–967.
Indyk H.E., Woollard D.C., 1995. Enzymatic determination of free carnitine in milk and infant formula. J. AOAC International, 78, 69–74.
Indyk H.E., Woollard D.C., Woollard G.A., 1997. Enzymatic determination of carnitine in milk and infant formula. Food Chem., 59, 325–332.
Janssens G.P., Rycke H., Hesta M., Wilde R.O.M., 1999. Analysis of carnitine, betaine, γ-butyrobetaine, and separate short-chain acylcarnitines in pigeon plasma, crop milk and tissues by HPLC coupled with UV-detection. Biotechnol. Tech., 12, 231–234.
Kempen T.A., Odle J., 1992. Quantification of carnitine esters by high-performance liquid chromatography. Effect of feeding medium-chain triglycerides on the plasma carnitine ester profile. J. Chromatogr., 584, 157–165.
Knüttel-Gustavsen S., Harmeyer J., 2007. The determination of L-carnitine in several food Samales. Food Chem., 105, 793–804.
Lamhonwah A.M., Wong J., Tam C., Mai L., Tein I., 2009. Organic cation/carnitine transporter family expression patterns in adult murine heart. Pathol. Res. Pract., 205, 395–401.
Li B.U.K., Bummer P. M., Hamilton J.W., Gudjonsson H., Zografi G., Olsen W.A., 1990. Uptake of L-carnitine by rat jejunal brush border microvillous membrane vesicles. Digest. Dis. Sci., 35, 333–339.
Minkler P.E., Brass E.P., Hiatt W.R., Ingalls S.T., Hoppel C.L., 1995. Quantification of Carnitine, Acetylcarnitine, and Total Carnitine in Tissues by High-Performance Liquid Chromatography: The Effect of Exercise on Carnitine Homeostasis in Man. Anal. Biochem., 231, 315–322.
Minkler P.E., Ingalls S.T., Hoppel C.L., 2005. Strategy for the Isolation, Derivatization, Chromatographic Separation, and Detection of Carnitine and Acylcarnitines. Anal. Chem., 77, 1448–1457.
Patkowska-Sokoła B., Bodkowski R., Aniołowski K., 2003. The content of L-carnitine in products of different animal species. Chemistry for Agriculture, vol. 4, 190–194.
Pękala J., Patkowska-Sokoła B., Bodkowski R., Jamroz D., Nowakowski P., Lochyński S., Librowski T., 2011. L-Carnitine – metabolic function and meaning in humans life. Curr. Drug Metab., (Epub ahead of print).
Ramsay R.R., Gandour R.D., van der Leij F.R., 2001. Molecular enzymology of carnitine transfer and transport. Biochim. Biophys. Acta, 1546, 21–43.
Rinaldo P., Stanley C.A., Hsu B.Y.L., Sabchez L.A., Stern H.J., 2002. Sudden neonatal death in carnitine transporter deficiency. J. Pediatr., 131, 304–305.
Seim H., Ezold R., Kleber H.P., Strack E., 1980. Stoffwechsel des L-carnitins bei Enterobakterien. Z. Allg. Mikrobiol., 20, 591–594.
Shoji Y., Koizumi A., Kayo T., Ohata T., Takahashi T., Harada K., Takada G., 1998. Evidence for linkage of human primary systemic carnitine deficiency with D5S436: A novel gene locus on chromosome 5q. Am. J. Hum. Genet., 63, 101–108.
Vaz F.M., Wanders R.J., 2002. Carnitine biosynthesis in mammals. Biochem. J., 361, 417–429.

7

THE EVALUATION OF AN ANTIOXIDANT ACTIVITY OF TEA, ROOIBOS AND SPINACH EXTRACTS AND THE DETERMINATION OF POLYPHENOLS IN PLANT LEAVES BY MEANS OF HPLC AND SPECTROPHOTOMETRY TECHNIQUES

Introduction

The edible plant material contains various groups of biologically active compounds such as polyphenols, vitamins, proteins, organic acids, sugars, glicosides among others [Del Rio et al. 2004, Pan et al. 2003, Weisburger 1997]. Flavonoids, anthocyanins, catechins and other belong to the polyphenols group. Numerous flavonoids have been identified (about 4000) and are found in plants (leaves and fruits), herbs and products such as wines, beers, juices, etc., but their presence in mosses and liverworts and even their occurrence in algae has been reported [Andersen and Markham 2006]. Many flavonoids occur in fruits, vegetables and beverages (tea, coffee, wine). They are classified into classes according to chemical structure [Andersen and Markham 2006, Grotewold 2006, De Beer et al. 2003]. The important roles of flavonoids are the capillary protective effect and antioxidant activity, the modulation of enzymatic activity, insect attraction or repulsion, nectar guides, viral, fungal, and bacterial protection, etc. Flavonoids are antioxidants and that reason they scavenge free radicals in organisms and protect most important biomolecules such as DNA, proteins, lipids from their damaging activity.

Also, carotenoids indicate the antioxidant actions based on their singled oxygen quenching properties and ability to trap peroxyl radicals [Paiva and Russell 1999, Stahl and Sies 1996]. The first one depends on the number of conjugated double bonds of the molecule and is influenced to a lesser extent by cyclic or acyclic carotenoid groups [Paiva and Russell 1999, Krinsky 1998]. Also, the nature of substituents in carotenoids are containing cyclic end group. One of the most efficient single oxygen quenchers is lycopen, a natural carotenoid contains eleven conjugated and two nonconjugated double bonds [Krinsky 1998]. Moreover, β-carotene is a scavenger of peroxyl radicals, mainly via singlet oxygen tension [Burton and Ingold 1984]. The interaction of carotenoids with peroxyl radicals previously needs present of unstable β-carotene radical adduct [Burton and Ingold 1984, Rice-Evans et al. 1997]. These adduct radicals can shown to be highly resonance stabilized and are predicted to be relatively unreactive. Afterwards, they may undergo decay to generate nonradical products and

may terminate radical reactions by binding to the attacking free radicals [Paiva and Russell 1999, Rice-Evans et al. 1997]. Carotenoids are destroyed during the reaction with peroxyl radicals. This is a confirmation of antioxidant activity of carotenoids group of compounds (Woodall et al. 1997).

Many assays have been introduced for the measurement of the total antioxidant activity of pure compounds [Re et al. 1999, Rice-Evans et al. 1996, Miller and Rice-Evans 1994, Miller et al. 1996, Kono et al. 1995, Arnao et al. 1990]. Each method relates to the generation of a different radical, acting through a variety of mechanisms and the measurement of a range of end points at a fixed time point or over a range (Rice-Evans et al. 1996, Miller and Rice--Evans 1994, Miller et al. 1996, Kono et al. 1995, Arnao et al. 1990). Two types of approach have been taken in the consideration. One of them is the inhibition assays in that the extent of the scavenging by hydrogen-or electron-donation of a pre-formed free radical is the marker of antioxidant activity, as well as assays involving the presence of antioxidant system during the generation of the radical [Re et al. 1999]. Mentioned assays have been based on different principles and experimental conditions. In general, DPPH• (1,1-diphenyl-2-picrylhydrazyl) and ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation forms the basis of one of the spectrophotometric methods have been applied to the measurement of the total antioxidant activity of solutions of pure substances. The total antioxidant activity can be expressed by the FRAP assay (ferric reducing antioxidant power), ORAC (oxygen radical absorbance capacity), superoxide radical-scavenging activity, TEAC (Trolox equivalent antioxidant activity) [Bertoncelj et al. 2007].

The violet-coloured free stable radical DPPH• is proved to be useful in a variety of investigations including an antioxidant properties of natural compounds as well as plant extracts [Ionita 2005]. This regent presents as a dark-coloured crystalline powder composed of stable free-radical molecules. DPPH is a well-known radical scavenger for other radicals. Therefore, the rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction. Because of a strong absorption band centre at about $\lambda = 520$ nm, the DPPH$^{\bullet}$ radical has a deep violet colour in solution, and it becomes colourless or pale yellow when neutralized. This property allows visual monitoring of the reaction, and the number of initial radicals can be counted from the change in the optical absorption of DPPH$^{\bullet}$. The scheme of reaction with DPPH$^{\bullet}$ is presented in Figure 1.

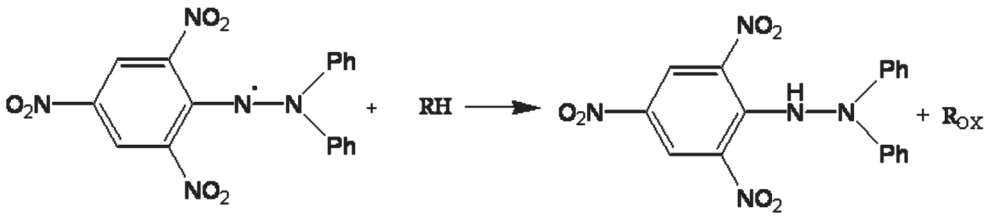


Fig. 1. Scheme of reaction with DPPH$^{\bullet}$

The antioxidant activity was expressed in RSA (radical scavenging activity). RSA was measured by using the following equation (1):

$$RSA = \frac{A_{DPPH} - A}{A_{DPPH}} \times 100\% \quad (1)$$

where:
A_{DPPH} – an absorbance values measured for blank DPPH•,
A – an absorbance values measured for samples (time reaction 30 min.).

The main aim of presented work was to investigate the effect of polyphenols concentration in plant leaves on the antioxidant activity of extracts obtained from examined plants. This study was carried out to determine the total antioxidant activity of selected plant leaves like tea, rooibos and spinach. Method for the determination of antioxidant activity is based on reaction between DPPH• and separated compounds from extracts of various sorts of plants. As a result – bleaching out of free radical from purple to yellow is obtained and measured. The results are expressed as rutin equivalents (RE) in mg per dry weight (g) of leaves. Antioxidant activity of gallic acid, catechin, rutin and total antioxidant activity were evaluated. For total antioxidant activity determination all peaks of the mirror chromatogram were integrated and expressed in RE. For the antioxidant activity evaluation in methanolic extracts of black and green tea (*Camellia sinensis*) and rooibos (*Aspalathus linearis*) a novel convenient on-line reaction detection assay with DPPH• requires HPLC instrumentation was applied. Major compounds such as: gallic acid, theobromine, epigallocatechin, catechin, caffeine and rutin, were identified in black, green teas and rooibos plant.

Next investigations confirmed that leaves of green vegetables like spinach (*Spinacia L.*) are a rich source of carotenoids including xanthophylls. The increased colouration in vegetable and fruit tissues associated with maturity is often indicative of increases in especially carotenoids concentrations. Carotenoids indicate the antioxidant actions based on their singled oxygen quenching properties and ability to trap peroxyl radicals. For that reason the total antioxidant activity of extracts from raw and cooked spinach leaves was evaluated.

Materials and Methods

Qualitative and quantitative analyses of tea samples by HPLC

Black and green teas, Rooibos were purchased in Polish local markets. 99% methanol (G.R., Lachema, Czech Republic) was used for the extraction of plant materials. Ammonium acetate (Merck, Germany), formic acid, analytical grade acetonitrile (MeCN), gallic acid, theobromine (Sigma-Aldrich, Germany), caffeine (Reag. Ph Eur, Merck, Germany) and catechin (Sigma-Aldrich, Germany) were used for HPLC analysis. The following reagents were used for the evaluation of radical scavenging and antioxidant activity: Na_2HPO_4 and NaH_2PO_4 (Merck, Germany), synthetic free radical DPPH• (Sigma-Aldrich, Germany), rutin hydrate, min 95 % HPLC (Sigma-Aldrich Chemie, Steinheim, Germany). The determination of antioxidants in tea was performed by a highperformance liquid chromatography (HPLC) system, which consisted of a model 1 100 quaternary pump (Hewlett Packard, Waldbronn, Germany), a model 1100 vacuum degasser (Hewlett Packard, Waldbronn, Germany), an autosampler (Perkin Elmer, USA), a precolumn, an analytical column ODS C-18 dp=5 μm reversed-phase 250 mm × 4.6 mm (Altech, Germany); the detection was carried out using a UV detector "Spectra 200" (Spectra Physics, Mountain View CA, USA); the buffered DPPH• solution was introduced to the system by a syringe pump (Phoenix 20 CU, Carlo Erba Instruments, Milan, Italy); the radical scavenging reaction was performed in the 0.75 m reaction coil (0.3 mm I.D.) and the signal of an antioxidant action was detected by a visible light detector "Linear UVIS 200" (Linear Instru-

ments, Reno, USA). Two channel signals were collected, calculated, and stored by ChromStar 3.20 software (Bruker, Bremen, Germany). One, at wavelength of 270 nm, is an image of tea compounds separation, and the other – the negative signal at wavelength of 517 nm, depicts the antioxidant activity. Before extraction, each tea sample was ground in a ceramic mortar; then 0.25 g of the ground tea was extracted with 2.5 ml of 100% methanol. All the extracts were stirred for half an hour, and then filtered using a 0.2 For HPLC, a separation gradient was used. Solvent A was 2.5 mM ammonium acetate in bidistilled water, which was acidified with formic acid (0.4 ml/l). HPLC grade MeCN mixed with solvent A in a volume ratio of 1:1, was used as solvent B. The percentage of solvent B in the gradient started from 20% and was as follows: 0–10 min from 20 to 30%, then to 40% in 10 min, hold for 4 min, then from 40 to 90% in 9 min and to 100% in 5 min. The flow rate was 0.4 ml×min^{-1}. The DPPH reagent was dissolved in MeCN at the concentration of 10 mg×L^{-1}. This solution was mixed with 50 mM phosphate buffer in a ratio of 1:2. The phosphate buffer was prepared of Na_2HPO_4, and pH = 7.6 was adjusted by adding NaH_2PO_4. The stream of the buffered DPPH• solution was introduced to the system at the flow rate of 0.4 mL×min^{-1}.

The flavonoids were separated on a 250 mm × 4.6 mm i.d., 5-μm particle, Hypersil ODS C_{18} column (Alltech, Lexington, Kentucky, USA). ChromStar 3.20 (Brucker, Germany) software was used to analyze the results.

Development of Chromatograms

Standard solutions and plant extracts were chromatographed at a flow rate of 0.4 mL min^{-1} (10 μL injection volume) by use of binary mobile phase gradient prepared from 2.5 mM ammonium acetate (pH 3) (component A) and 50:50 (v/v) ammonium acetate–acetonitrile (pH 3) (component B). The gradient was: 0 min, 20% B; 10 min, 30% B; 20 min, 40% B; 24 min, 40% B; 35 min, 90% B; 40 min, 100% B. Flavonoids were monitored by UV absorbance at 231 and 517 nm. All chromatography was performed at 25°C.

Quantitative analysis was performed by HPLC with the conditions described above. For calibration, mixed standard solutions of rutin, catechin, and gallic acid in the range 0.020 – 0.500 mg×mL^{-1} were prepared in methanol. Real tea extracts were also analyzed. Before extraction, each tea sample was ground in a ceramic mortar. The ground tea (250 mg) was then extracted with 2.5 mL 100% methanol. All extracts were stirred for 30 min then filtered through a 0.2-μm membrane filter.

Conditions for the preparation of spinach extracts

Samples of fresh and frozen spinach were obtained from local markets. Three samples of frozen spinach (marking I, II, III) came from various manufacturers of frozen food. Previously, samples of frozen spinach were kept at room temperature during 3 h. Next spinach leaves were dried during 8–24 h at temperature 40ºC. Afterwards plant material was grounded in mortar with pestle. Obtained samples (0.5 g, fraction 0.1–0.2 mm) of leaves were mixed with 2 mL of methanol. Samples were shaken occasionally during 24 hours. The extracts were then filtered to remove solid plant debris, and kept in a refrigerator (4ºC).

Evaluation of antioxidant activity of spinach extracts

Method is based on reaction between DPPH$^{\bullet}$ and separated compounds from extracts of mentioned plant leaves. As a result – bleaching out of free radical from purple to yellow

is obtained and measured. The results are expressed as rutin equivalents (RE) in mg per dry weight (g) of tea. Antioxidant activity of gallic acid, catechin, rutin and total antioxidant activity were evaluated. For total antioxidant activity determination all peaks of the mirror chromatogram were integrated and expressed in RE.

Determination of total phenolics contents in spinach extracts

As it is known the total phenolics content are measured spectrophotometrically at λ=760 nm using the Folin–Ciocalteu reagent. This reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of polyphenols. Standards of gallic acid in concentration range from 0.1 to 1.3 mg×mL^{-1} were also measured. The results were expressed as gallic acid equivalents (GAE) of dry matter.

Determination of total flavonoids contents in spinach extracts

The total flavonoids content was measured spectrophotometrically at 407 nm using hexamethyltetramin (5%), methanol, acetic acid (33%), distilled water and aluminum chloride as reagents. Reaction time 30 min. was applied. Standards of rutin in concentration range from 0.1 to 1.0 mg×mL^{-1} were also measured. The results were expressed as rutin equivalents (RE) dry matter.

Results and Discussion

In Figure 2 are shown HPLC-UV and HPLC-DPPH chromatograms of black, green and rooibos tea. The identification of gallic acid, theobromine, catechin, caffeine, and rutin peaks was made using standard solutions of these compounds. Epigallocatechin was identified from literature data [Milašienė et al. 2007, Bonoli and Lercker 2003, Yang and Jiang 2007] according to its elution order.

As the antioxidant activity was expressed in RE, calibration graph for rutin was constructed from data obtained using the DPPH$^{\bullet}$ reaction detector. The equation obtained was used to evaluate antioxidant activity of different compounds in RE (mg×g^{-1}), where: Peak_area expressed in mV×min^{-1} (Equation 2)

$$RE = \frac{Peak\_area}{3855.6} \tag{2}$$

The quantification results for some important compounds (gallic acid, epigallocatechin, catechin, and rutin) are shown in Table 1, and their quantities, as obtained using UV detection, are expressed in mg per 1 g of dry tea. The comparison of tea species showed that the highest amount of rutin appears in black tea, whereas its content is lower in green tea. Moreover, it was not determined in rooibos tea. Epigallocatechin was identified in the green tea chromatogram using the epigallocatechin standard solution. However, from the literature sources it is known, that green tea contains more catechin group compounds comparing to black tea. This is reflected in higher antioxidant activity of green tea. The highest amount of gallic acid appears in black tea, although its content in green and black tea is comparable.

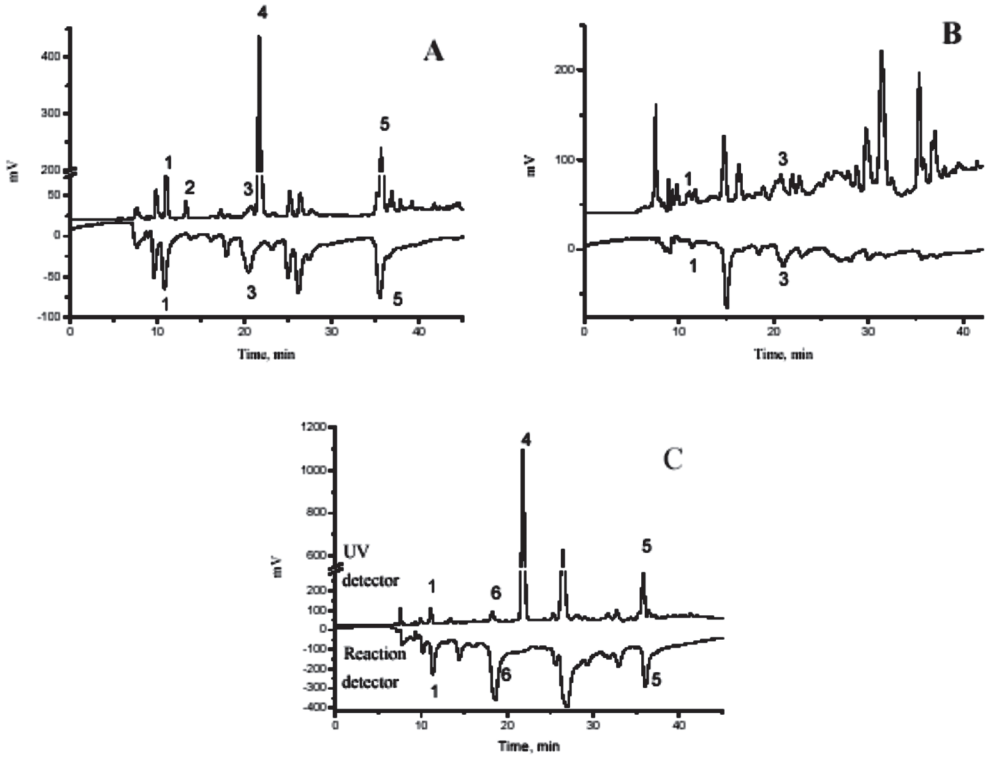


Fig. 2. Chromatograms of black tea extract (A), Rooibos extract (B), and green tea extract (C). Peaks: 1 – gallic acid, 2 – theobromine, 3 – catechin, 4 – caffeine, 5 – rutin, 6 – epigallocatechin

In Table 2 the antioxidant activity of main compounds i.e. gallic acid, catechins, and rutin in black, green and rooibos tea, determined using the reaction detection, is shown. The results indicate, that the amount of rutin, good correlation between quantitative analysis data (Tab. 1) and antioxidant activity (Tab. 2) was obtained for rutin. Higher content and higher antioxidant activity of this compound was in black tea. The same correlation is observed between the amount of gallic acid and its radical scavenging activity, i.e. it is also higher in the case of black tea. The content of catechin in black tea was higher comparing to epigallocatechin in green tea. The antioxidant activity expressed in mg/g RE, however, was higher for epigallocatechin in green tea comparing to catechin in black tea. The results obtained let us draw the conclusion, that epigallocatechin is a stronger antioxidant than catechin.

The total antioxidant activity was evaluated by summing up all the negative peak areas of the mirror chromatogram expressed in $mg \times g^{-1}$ RE. The results obtained are shown in Table 3. The total antioxidant activity varied from 4.29 $mg \times g^{-1}$ to 24.19 $mg \times g^{-1}$ and was highest for green tea and lowest for rooibos tea. This can be explained by the fact that unfermented green tea has a higher content of catechin group compounds, which are stronger radical scavengers.

Table 1

Results of quantitative analyses of rutin, catechin, and gallic acid in black tea, green tea, and Rooibos extracts (n=3)

	Concentration [mg×mL^{-1}]			Concentration [mg×g^{-1}]			Concentration [% w/w]		
	Rutin	Catechin	Gallic acid	Rutin	Catechin	Gallic acid	Rutin	Catechin	Gallic acid
Black tea	1.009 ± 0.005	0.462 ± 0.008	0.191 ± 0.005	10.090 ± 0.015	4.624 ± 0.100	1.907 ± 0.065	1.01	0.46	0.19
Green tea	1.025 ± 0.008	1.290 ± 0.012	0.156 ± 0.001	10.250 ± 0.096	12.903 ± 0.118	1.560 ± 0.015	1.03	1.29	0.16
Rooibos	nd	0.111 ± 0.002	0.011 ± 0.001	nd	1.112 ± 0.020	0.105 ± 0.001	nd	0.11	0.01

Table 2

Antioxidant activity of gallic acid, catechins and rutin in black, green tea and Rooibos extracts determined by using of HPLC-DPPH method

Sample	Antioxidant activity, mg×g^{-1} RE			
	Gallic Acid	Catechin compounds		Rutin
		Catechin	Epigallocatechin	
Black tea	3.12	3.16	–	3.29
Green tea	2.41	–	4.56	2.65
Rooibos	0.11	0.91	–	0.00

Table 3

Total radical scavenging activity for black, green tea and Rooibos extracts determined by using of HPLC-DPPH

Sample	Antioxidant activity, mg×g^{-1} RE
Black tea extract	18.71
Green tea extract	24.19
Rooibos 100 % methanol extract	4.29

During our studies the antioxidant activity of fresh and frozen spinach extracts was examined by means of classical spectrophotometry. Besides one sample we obtained higher results of RSA for frozen spinach than for fresh spinach. RSA values for frozen spinach were respectively obtained 16.2, 30.3 and 42.6%. Otherwise, RSA obtained for fresh spinach did not exceed 19.8%. Particular results obtained during experiments concern the determination of RSA for fresh and frozen spinach extracts are presented in Figure 3.

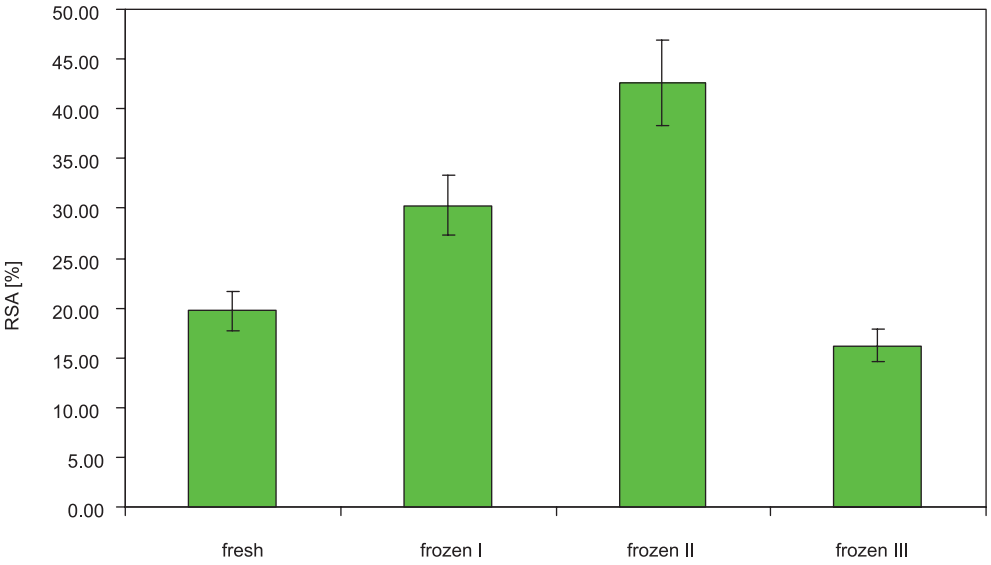


Fig. 3. The antioxidant activity of extracts from spinach

During plant leaves development and expansion, pigmentation increases to provide energy through photosynthesis. The physiological age of leaves directly influences colouration and energy production within the plant (leaves, fruits and rots), which is a result of changes in chlorophyll, carotenoid pigments and polyphenols concentrations. The increased colouration in vegetable and fruit tissues associated with maturity is often indicative of increases in especially carotenoid concentrations [Lefsrud et al. 2007]. It is still unclear, what is the progress of changes of carotenoid concentration in leaves during leaf ontogeny and an influence of various cultivation conditions on carotenoids concentration.

Moreover, many compounds mentioned above influence the antioxidant activity of plant extracts. One of them is lutein, which is xantophyll and belongs to the carotenoids group. Radical scavenging activity was evaluated for lutein solutions in concentration range of substance from 10 to 100 mg×mL^{-1} (Fig. 4). The less concentration of lutein in the solution gave RSA value 6.2%, but for ten times higher concentration of lutein (100 mg×mL^{-1}), obtained values of RSA did not exceed 10.0%. Obtained results show that lutein as a component of spinach extracts takes an inconspicuous part of the total antioxidant activity of this plant extracts.

Many compounds including polyhenols, chlorophylls, as well as carotenoids influence the antioxidant activity of plant and natural products extracts. Particular results obtained after the determination of total polyphenols as a gallic acid (GAE) equivalents and total flavonoids

as rutin (RE) equivalents by used of colorimetric method are presented in Table 4. These data were obtained for plant and natural products extracts.

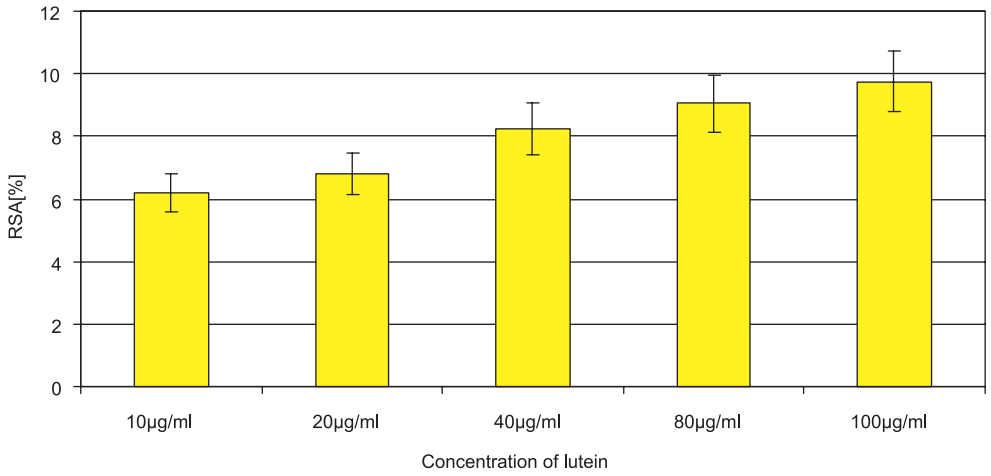


Fig. 4. The antioxidant activity of lutein standards

Table 4

Polyphenol acids and flavonoids present in samples of plants calculated from dry matter of sample

Sample	Concentration of polyphenols acids [mg GAE×g^{-1} dry matter]	Concentration of flavonoids [mg RE×g^{-1} dry matter]
Fresh spinach	1.823 ± 0.040	0.626 ± 0.013
Frozen spinach I	4.946 ± 0.098	0.370 ± 0.007
Frozen spinach II	4.586 ± 0.092	0.354 ± 0.012
Frozen spinach III	4.589 ± 0.098	0.262 ± 0.008

The total content of polyphenols in spinach samples varied from 1.823 to 4.946 mg×g^{-1} of dry matter. In presented study the lower concentration of flavonoids in all samples was observed.

Conclusions

The antioxidant activity of extracts from plants and natural products is correlated to the concentration of polyphenols including phenolic acids and flavonoids.

For the antioxidant activity evaluation in methanolic extracts of black and green tea (*Camellia sinensis*) and rooibos tea (*Aspalathus linearis*) a novel convenient on-line reaction detection assay with DPPH$^{\bullet}$ was used. The main compounds such as: gallic acid, theobromine, epigallocatechin, catechin, caffeine and rutin, were identified in black, green teas and Rooibos plant. In general, green tea has been found superior to black tea in terms of its antioxidant activity owing to the higher catechins content the value of which is approximately 30% higher when compared to black tea. Rooibos tea showed a lower antioxidant activity.

Catechin group compounds (epigallocatechin etc.) provide a higher radical scavenging activity than gallic acid in it. The amount of rutin and gallic acid in black tea is approximately 20% higher comparing to green tea.

Among vegetables total antioxidant activity of spinach is relatively high. The high level of the content of polyphenol acids and flavonoids in spinach leaves influence the high the antioxidant activity.

Acknowledgements

Investigations were supported by Sokrates-Erasmus scholarships for K. Sawicka, N. Charczun, Ł. Starosta.

The authors wish to thank Judyta Piotrowska, MSc for her technical assistance.

References

Andersen Ř.M. and Markham K.R., 2006. Flavonoids. Chemistry, Biochemistry and Applications. CRS Press, Taylor & Francis Group, Baca Raton, Finland.

Arnao M.B., Casas J.L., del Rio J.A., Acosta M. and Garcia-Canovas F., 1990. Anal. Biochem., 185, 335–338.

Bertoncelj J., Doberšek U., Jamnik M. and Golob T., 2007. Food Chem., 105 (2), 822–828.

Bonoli M. and Lercker G., 2003. Food Chem., 81, 631–638.

Burton G.W. and Ingold K.U., 1984. Science 224, 569–573.

De Beer D., Joubert E., Getderblom W.C.A. and Manley M., 2003. J. Agric Food Chem., 51 902–909.

Del Rio D., Steward A.J., Mullen W., Burns J., Lean M.E.J., Brighenti F. and Crozier A., 2004. J. Agric. Food Chem. 52, 2807–2815.

Grotewold E., 2006. The Science of Flavonoids. Springer, Columbus, Ohio, USA.

Ionita P., 2005. Chem. Pap., 59(1), 11–16.

Kono Y., Shibata H., Kodama Y., Sawa Y., 1995. Biochem. J. 312, 947–953.

Krinsky N.I., 1998. Proc. Soc. Exp. Biol. Med., 218, 95–97.

Lefsrud M., Kopsell D., Wenzel A. and Sheehan J., 2007. Scientia Horticulturae, 112, 136–141.

Milašienė R., Sawicka K., Kornyšova O., Ligor M., Maruška A., Buszewski B., 2007. Ars Separatoria Acta, 5, 27–33.

Miller N.J. and Rice-Evans C.A., 1994. Methods Enzymol., 234, 279–293.

Miller N.J., Castelluccio C., Tijburg L. and Rice-Evans C.A., 1996. FEBS Letts., 392, 40–44.

Paiva S.A.R. and Russell R.M., 1999. Journal of the American College of Nutrition, 18(5), 426–433.

Pan X., Niu G. and Liu H., 2003. Chem. Eng. Proc., 40, 129–133.

Re R., Pellegrini N., Proteggente A., Pannala A., Yang M. and Rice-Evans C., 1999. Free Radic. Biol. Med., 26, 1231–1237.

Rice-Evans C.A., Miller N.J., Paganga G., 1996. Free Radic. Biol. Med., 20, 933–956.

Rice-Evans C.A., Sampson J., Bramley P.M., Holloway D.E., 1997. Free Radic. Res., 26, 381–398.

Stahl W., Sies H., 1996. Arch. Biochem. Biophys., 336, 1–9.

Weisburger J.H., 1997. Cancer Lett., 114, 315–317.

Woodall A.A., Britton G., Jackson M.J., 1997. Biochim. Biophys. Acta (BBA), 1336, 75–586.

Yang X.R. and Jiang Y.M., 2007. Food Chem., 100 (3), 1132–1136.

8

SIMPLE PHARMACOPHORE MODEL FOR THE PREDICTION OF BITTER TASTE OF CAFFEINE DERIVATIVES

Introduction

The human taste apparatus is constantly evolving in response to inevitable changes in the natural environment and factors related to the genetic inheritance of taste perception. It is determined by the type of available food and closely linked to age and individual taste preferences [Kikut-Ligaj, Jasiczak 2006, Stevens et al. 1988]. One of the main tastes is bitter taste. The reason why humans have a sense of that taste is to protect us from ingesting toxic substances, at the same time ensuring a necessary amount of natural medicines, metabolites, neuro- and psychostimulants.

Qualitative and quantitative studies of bitter taste, which we have been conducting using computer methods, make it possible to track the process of gustatory recognition of the active molecule (ligand) and thus predict its approximate taste activity [Kikut-Ligaj et al. 2010a, b, 2005, Kikut-Ligaj, Jasiczak 2004a]. Predicting bitter taste activity of natural and synthetic organic compounds (bitter ligands) is extremely important, for example in the process of developing flavour formulas for food products, nutraceuticals and pharmaceuticals [Kikut--Ligaj, Jasiczak 2006, Stevens et al. 1988].

The recent discovery of a range of bitter taste receptor proteins [Miguet et al. 2006, Kenji et al. 2008, Temussi 2009, Reed et al. 2006] has shown that the development of a universal and homogeneous model of bitter taste perception is not feasible. Bitter taste is recognized by a family of ca. ~ 25 bitter taste receptors (TAS2Rs) contained in gustatory cells [Behrens et al. 2007, Adler et al. 2000, Shi et al. 2006, Biarnés et.al. 2010]. TAS2Rs belong to the super-family of receptors that have seven transmembrane helices and interact with intracellular G proteins, which is why they are often referred to as heptahelical or G protein-coupled receptors (GPCRs).

The reports have prompted us to focus our research effort exclusively on small-molecule bitter ligands. We have assumed that the process of bitter taste detection of small--molecule bitter ligands involves a group of structurally similar receptor proteins (GPCRs). Within the group of these receptor proteins, sites activated by bitter ligands should be very similar in structural aspects. It cannot be ruled out that members of the above-mentioned family of receptor proteins can have a structurally similar "receptor pocket" form. This hypothesis can be corroborated, for example, by common features identifiable in the structure

of many small-molecule bitter ligands. It does not seem probable for each class of bitter ligands to have a different receptor protein – if this were the case, given the huge number of classes of bitter-tasting compounds, the recognition apparatus for bitter taste would be too complicated.

For bitter taste predictions we employ computer analysis to investigate the structure of bitter ligands in their steric and electronic aspects. A key stage in the identification of interactions between the ligand and its receptor protein is finding an appropriate pharmacophore referring to the spatial orientation of different functional groups (differing, for example, with regard to electron properties), necessary to trigger biological activity [Bielenica et al. 2010, Kikut-Ligaj, Jasiczak 2004b]. In actual fact, the pharmacophore describes the spatial arrangement of selected features of the molecular structure such as load densities, donor and acceptor sites of hydrogen bonds, hydrophobic groups and aromatic areas [Seidel et al. 2010]. In brief, the pharmacophore is a general 3D "molecular form" exposing repeatable properties in a specified set of active ligands [Martin 2007]. In many cases pharmacophore models fail to adequately account for subtle differences in the molecular structure of ligands which are responsible for the loss of biological activity. More details concerning the influence of specific areas of the ligand molecule on the ligand's biological activity can be obtained by QSAR (Quantitative Structure-Activity Relationships) [Lauria et al. 2010, Kikut-Ligaj, Jasiczak 2004b], CoMFA (Comparative Molecular Field Analysis) [Caballero 2010], 3D pharmacophore-based techniques [Wolber 2008, Seidel et al. 2010] or molecular docking [Lauria et al. 2010].

Due to their distinct spatial structure, we have taken an interest in the group of bitter ligands (methylxanthine alkaloids) including caffeine [Kikut-Ligaj, Jasiczak 2004b].

In order to explain how caffeine binds to the receptor protein, a series of structurally similar methylxanthine alkaloids were analyzed. The selected methylxanthine group was used for developing a simple pharmacophore model for this group of compounds. However, an analysis of pharmacophore models is not sufficient for determining all descriptors coding for bitter taste activity. Some of the descriptors are decoded and verified by means of methods based on docking into the so-called molecular mould (MM), which is extremely important due to the fact that even slight spatial modifications in the structures of bitter ligands can – either entirely or partially – mask the sensation of bitter taste [Kikut-Ligaj et al. 2010a, b, 2005, Kikut-Ligaj, Jasiczak 2004a]. Docking into the hypothetical (virtual) area of the receptor pocket represented by the molecular mould (MM) makes it possible to determine active regions (subregions) of the ligand and the number of spatial stimulations with which a single ligand activates a receptor protein. The mould is composed of four structurally different bitter ligands, such as caffeine, quinine, khellin and tetraiodosaccharin, superimposed on one another with areas exhibiting similar nucleophilic or electrophilic features [Kikut-Ligaj et al. 2010a, b, 2005; Kikut-Ligaj, Jasiczak 2004a].

Because of the simple design of the mould, hypothetical – and so far unexplored – taste ligands are quickly assessed to determine their bitter taste. By constructing a pharmacophore 3D model and docking the ligand into the 3D mould, we are able to establish mathematical descriptors coding for the bitter activity of a ligand.

For the purpose of improving our simple molecular mould, we have been conducting analyses of a dozen groups of bitter ligands [Nowotna-Kromolicka et al. 2007]. The studies will make it possible to determine approximate locations of active sites and sub-sites at the

time of ligand interaction with the receptor protein. Both methods of analyzing bitter taste activity (pharmacophore generation and MM docking) are mutually complementary, which makes the research apparatus more precise.

Materials and methods

Compounds and biological data

Methylxanthines belong to a group of plant-derived compounds which are commonly found in food products at various degrees of processing. They are also ingredients of nutraceuticals and pharmaceuticals. The methylxanthine caffeine is present in a number of plant species (e.g. coffee, tea, guarana, yerba mate) which use it – as a natural pesticide – for combating insects. The effects of caffeine (similarly to many other methylxanthines) on the human body are multidirectional and still not fully known [Nehlig et al. 1992]. For example, many methylxanthines act on the respiratory and circulatory systems, as well as the central nervous system.

Methylxanthine alkaloids used in the analyses were purchased from Sigma-Aldich. All the compounds used were of HPLC purity. We examined the following compounds: caffeine (**1**), theophylline (**2**), 7-(2,3-dihydroxypropyl) theophylline (**3**), theophylline-8-propionic acid (**4**), theophylline-7-acetic acid (**5**) and theobromine (**6**) (see Fig.1).

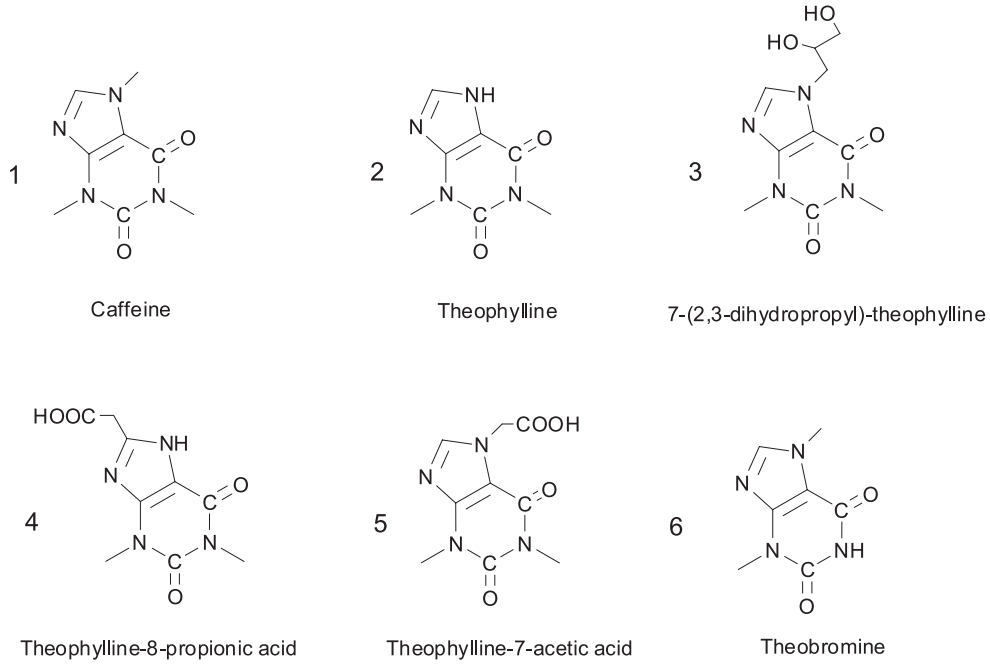


Fig. 1. Structures of compounds under study (1–6)

Sensory analysis

Sensory analyses were carried out, based on the Polish Standards, using the *sip-and--spit* technique (i.e. rinsing the mouth with aqueous solutions of the alkaloids under study) [Kreppenhofer et al. 2011, Stark et al. 2005, Frank et al. 2007, 2008, Delwiche et al. 1996, Klimacka-Nawrot et al. 2007, 2008). The object of analyses were aqueous solutions of compounds 1–6 at above-threshold concentrations (0.002 g/100 ml water). According to rules adopted for analyses of this type (PN-ISO 8586-1(2), 1998), the group of testers (assessment team comprising individuals with proven sensory sensitivity) was carefully selected and properly trained. The testers were tasked with ordering the compounds by the intensity of their bitter taste. Sensory tests were conducted by a group of 15 testers (PN-ISO 8586-1(2), 1998; PN-ISO 8589, 1998; PN-ISO 6658, 1998). Each test sample was performed in three replicates.

Results obtained in the study were represented in a scale comprising a numerical range (0-4) and descriptive categories (i.e. not bitter, slightly bitter, moderately bitter, bitter and very bitter). Experimental data gathered in the analysis were then processed statistically.

Computer methods used in analyses of compounds 1–6

All computer calculations were performed in the Gaussian 09W application. The geometry of caffeine and the other methylxanthine alkaloids studied (1–6) was optimized by means of DFT (*density functional theory*) using the B3LYP functional with the 6–31G(2d,p) basis set [Kim et al. 2001]. The presence of water as a solvent was stimulated with the CPCM (*Conducting Polarized Continuum Model*) model [Takano et al. 2005, Zhan Chen et al. 2010, Nunes et al. 2008]. The compounds under study, similarly to the range of previously examined substances [Kikut-Ligaj et al. 2010a, b, 2005, Kikut-Ligaj, Jasiczak 2004a], were docked into the 3D molecular mould model using the Chem-X application.

Results and Discussion

3D pharmacophore model creation

Pharmacophore is a model describing spatial relations between elements that are common for ligands interacting with a particular receptor [Bielenica et al. 2010]. According to a detailed definition, pharmacophore is an ensemble of steric and electronic properties which are necessary to ensure optimal supramolecular interactions with a specific biological target structure (receptor) [Bielenica et al. 2010]. The process of creating a simple pharmacophore for a group of selected methylxanthine alkaloids is presented in the figure below.

The analysis involved three bitter ligands (**1**, **2**, **6)** and three potentially bitter ligands (**3**–**5**) belonging to the group of methylxanthine alkaloids. The pharmacophore model applied for the analysis contains three areas which are key in the recognition of the studied methylxanthine alkaloids by a specific receptor protein. The three-point pharmacophore model contains nucleophilic sites located on carbonyl groups (C_2=O and C_6=O) and on the tertiary nitrogen atom N_9. Because of the flat methylxanthine skeleton of ligands **1–6**, the 2D pharmacophore model (Fig. 2) is at the same time a 3D model. Distances between the sites are specified in Table 1.

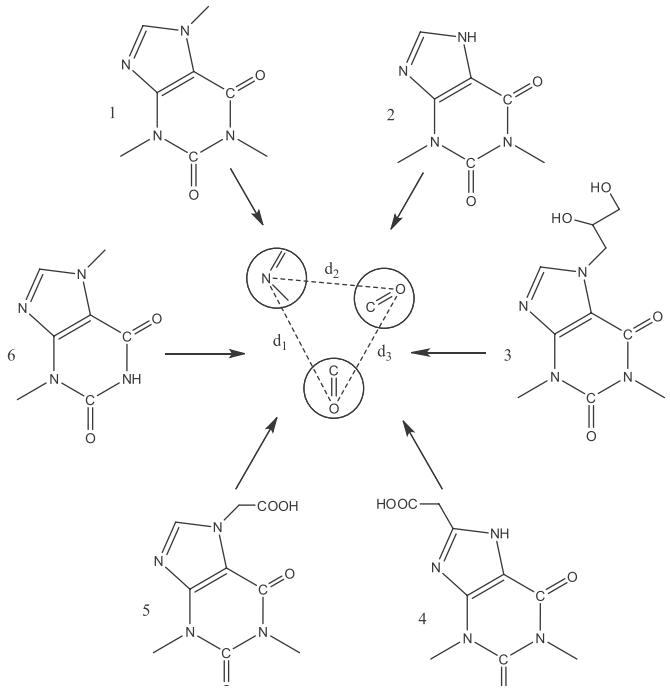


Fig. 2. Three-point pharmacophore model for the bitter taste receptor

Table 1

Distances between nucleophilic active sites for the methylxanthine alkaloids under study

No.	Compound	Distance (nm)		
		d_1(nm)	d_2(nm)	d_3(nm)
1	Caffeine	0.476	0.468	0.466
2	Theophylline	0.467	0.458	0.454
3	7-(2,3-dihydroxypropyl) theophylline	0.469	0.460	0.457
4	Theophylline-8-propionic acid	0.477	0.467	0.467
5	Theophylline-7-acetic acid	0.476	0.467	0.466
6	Theobromine	0.467	0.460	0.451
		$\overline{d}_1$=0.472	$\overline{d}_2$=0.463	$\overline{d}_3$=0.460

Distances listed in Table 1 above were recorded for structures **1–6** optimized by DFT (B3LYP/6-31G(2d,p)) in water. Mean distances $\overline{d}_1$, $\overline{d}_2$ and $\overline{d}_3$ (Table 1) between the studied sites for the generated pharmacophore model suggest symmetric distribution of electronic traits for all alkaloid structures under analysis.

Description of active sites of caffeine and structures 2–6

A detailed analysis of active sites of bitter ligands is crucial for their correct docking into the molecular mould [Jasiczak et al. 2007]. An analysis of the structure of ligands allows

for differentiation of their receptor binding sites and specification of their nucleophilic and electrophilic features.

As the first stage of analysis, the description of molecular charge distribution was prepared for individual nucleophilic regions of compounds **1–6**. A list of molecular charges on oxygen atoms in carbonyl groups C_2=O and C_6=O and on the tertiary nitrogen atom N_9 is presented in Table 2.

Table 2

List of molecular charges for the active sites of compounds under analysis

Compound no.	Molecular charge on the oxygen atom C_2=O (active site B_1)	Molecular charge on the oxygen atom C_6=O (active site B_2)	Molecular charge on the nitrogen atom N_9 (active site B_3)
1	-0.483	-0.482	-0.423
2	-0.446	-0.437	-0.389
3	-0.462	-0.455	-0.419
4	-0.484	-0.483	-0.454
5	-0.480	-0.480	-0.421
6	-0.444	-0.426	-0.420

The sites enumerated above are dominant in terms of negative molecular charge. The analysis made it possible to assign to the caffeine molecule (1) three nucleophilic active sites B_1, B_2 and B_3 located on carbonyl groups (C_2=O and C_6=O) and on the tertiary nitrogen atom N_9 (Fig. 3).

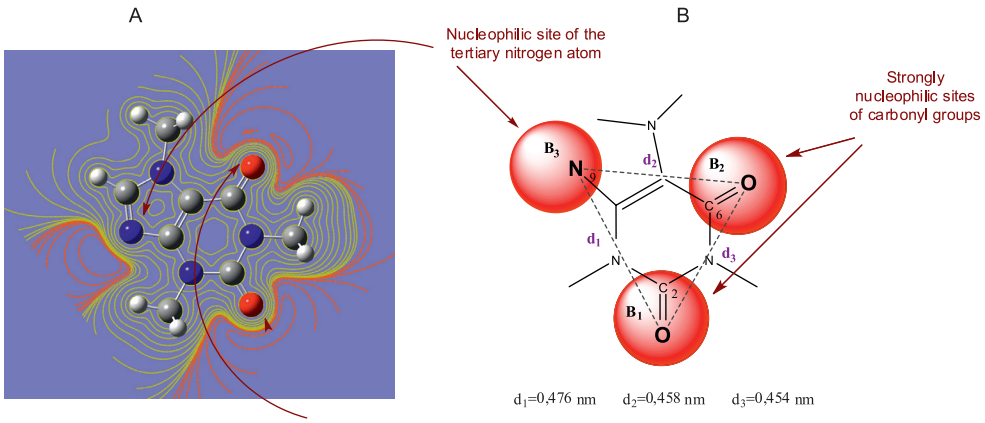


Fig. 3. Map illustrating the distribution of molecular charge densities around the caffeine molecule (A) along with the spatial arrangement of nucleophilic active sites (B)

In the course of analysis of the proposed nucleophilic sites (**B_1**, **B_2** and **B_3**) (Fig. 3) it was found that they were easily available for interactions with the receptor protein. The process is facilitated by two typical elements of the structure of caffeine and compounds designated as **2-6**, i.e. flat xanthine skeleton and symmetry observed in the mutual arrangement of sites **B_1**, **B_2** and **B_3**. The symmetric distribution of nucleophilic sites **B_1**, **B_2** and **B_3** is corroborated by

comparable distances d_1, d_2 and d_3 (cf. Tab. 1). Due to equidistant distribution of nucleophilic active sites the caffeine molecule is able to easily fit into the active area of the receptor protein because it is not important which area of the ligand approaches the receptor. The equivalence of sites $\mathbf{B_1}$, $\mathbf{B_2}$ and $\mathbf{B_3}$ is also clearly visible in Fig. 4 which visually represents the superimposition of all active sites for the caffeine molecule.

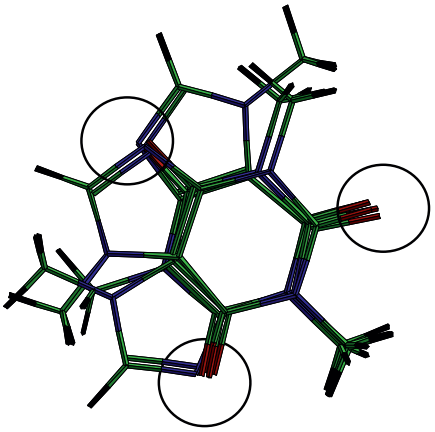


Fig. 4. Superimposition of active sites for the caffeine molecule

The properties of nucleophilic sites B_1, B_2 and B_3 enumerated above affect the speed of interactions between the receptor protein and the bitter ligand, making their reaction faster and stronger, and thus increasing the intensity of taste.

Molecular docking in taste analysis. Computer projections of the methylxanthine alkaloids into the molecular mould of the bitter taste receptor

Docking into the molecular mould is very important for the visualization of all possible fittings of a given molecule into the hypothetical area of receptor recognition. Because of its limited 3D form, the mould only allows molecules with a strictly defined spatial orientation and geometry of active sites. Consequently, every bitter ligand has a specific number of active sites (***NPI*** – number of point interactions) and a specific number of possible fittings into the receptor recognition area (***NIS*** – number of interactive stimulations). ***NPI*** and ***NIS*** are descriptors coding for the bitter taste of ligands. In order to determine these descriptors, computer projections into the MM area were performed for the ligands under study.

Computer projections of caffeine into the molecular mould

In view of its structure, caffeine has four possibilities (***NIS***=4) of fitting into the receptor recognition area (represented by the mould) (cf. Figs. 5a–d). Point interactions with three (***NPI***=3) nucleophilic active sites are observed at the boundaries of sectors D/A, A/B and C/D.

Computer projections were also performed for the other compounds (not presented in this article) (i.e. 2–6), for which ***NIS*** and ***NPI*** parameters were recorded (Tab. 3).

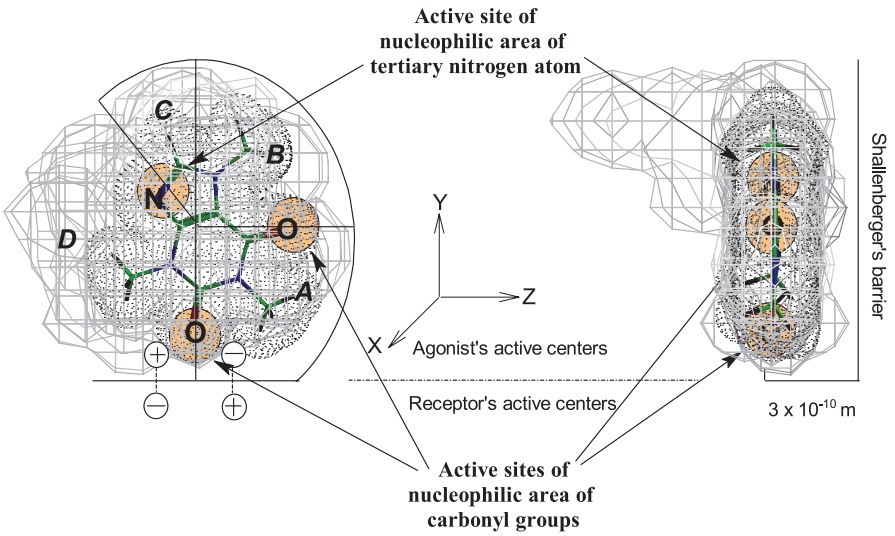


Fig. 5a. Caffeine molecule docked with the active site located on the oxygen atom of the carbonyl group C_2=O

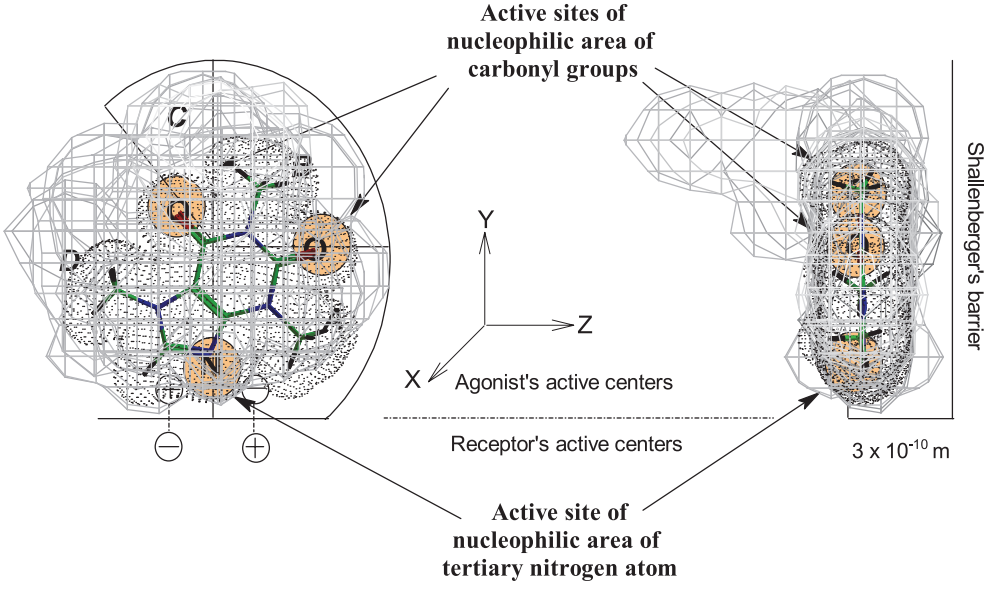


Fig. 5b. Caffeine molecule docked with the active site located on the nitrogen –N_9= atom

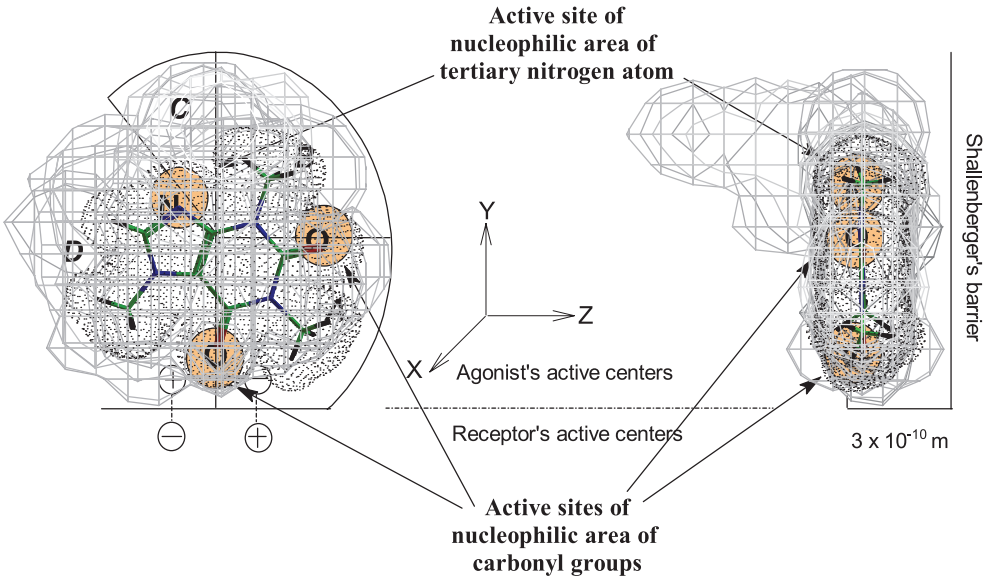


Fig. 5c. Caffeine molecule docked with the active site located on the oxygen atom of the carbonyl group C_6=O

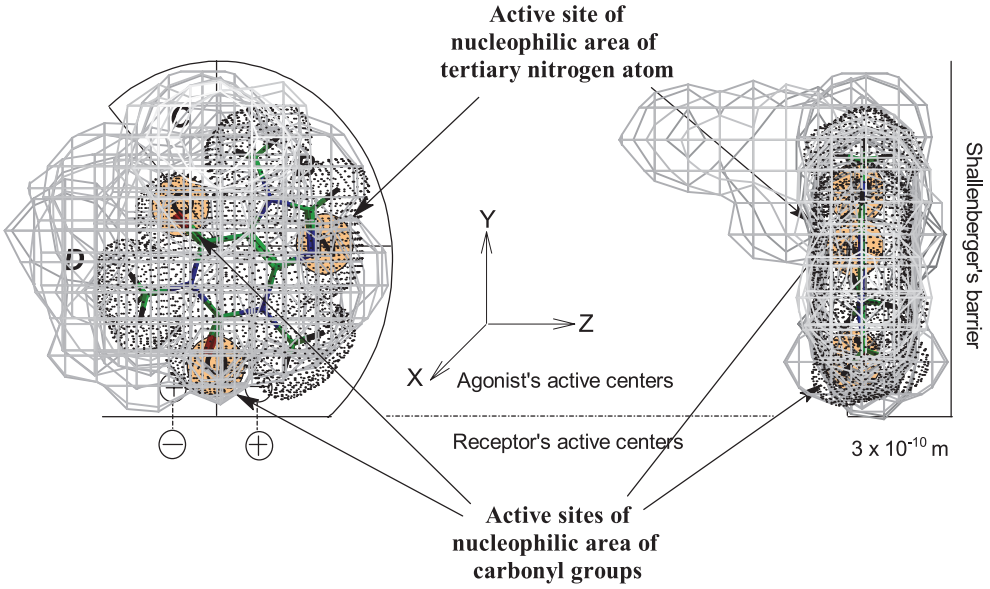


Fig. 5d. Caffeine molecule docked with the active site located on the oxygen atom of the carbonyl group C_2=O

Table 3

Predicted bitter taste index (BTI) and determined values of *NIS* and *NPI* parameters for the methylxanthine alkaloids under study

Compound no.	*NPI*	*NIS*	BTI= ∑ NPI + ∑ NIS
1	3	4	7
2	3	6(5)	9
3	3	3	6
4	3	4	7
5	3	3	6
6	3	4	7

The predicted value of the bitter taste index (BTI), as presented in the table above, is a sum total of the ***NIS*** and ***NPI*** parameters (BTI= ∑ ***NPI***+ ∑ ***NIS***).

The highest predicted value, BTI=9, was obtained for theophylline (2). Caffeine (1), as well as theobromine (6) and theophylline-8-propionic acid (4), exhibit moderate values of the bitterness index (BTI=7). The remaining compounds from the investigated group of methylxanthine alkaloids (3 and 5) should be the least bitter (BTI=6). The values of ***NIS*** and ***NPI*** descriptors, and the taste equation they express, demonstrate minor differences, which seems to suggest that the chemical compounds under analysis have a similar intensity of bitter taste.

Results of sensory analyses and their relation to the predicted taste of compounds 1–6

Sensory analysis was carried out to review values obtained for the predicted bitter taste of compounds **1-6**. The task of "the testers" was to assess whether aqueous solutions of the alkaloids (**1-6**) are bitter and to estimate their potential taste intensity. Taste activity was assessed using a five-grade hybrid scale (a combination of descriptive categories and a numerical scale) (not bitter-0, slightly bitter-1, moderately bitter-2, bitter-3 and very bitter-4). Results of analyses are listed in Table 4 below.

Table 4

Bitter Taste Index (BTI) for the methylxanthine alkaloids under study (1–6)

Compound no.	Descriptive scale	Numerical scale
0 (water)	not bitter	0
1	moderately bitter	2
2	bitter	3
3	slightly bitter	1
4	moderately bitter	2
5	moderately bitter	2
6	bitter	3

All the testers regarded the compounds as bitter-tasting. Sensory analyses, according to predictions (computer modelling) (Fig. 6), showed that the substances did not differ markedly in terms of taste intensity.

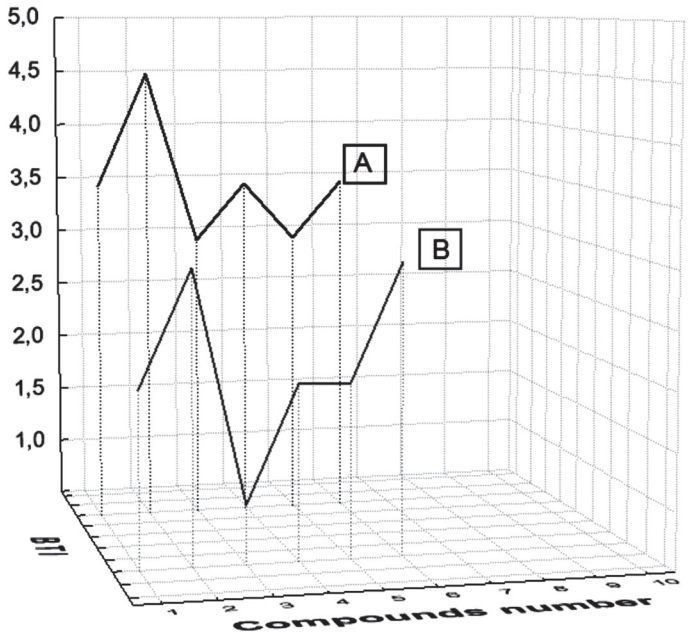


Fig. 6. Graph presenting BTI values for compounds 1-6 that were predicted (A) and determined by sensory analysis (B)

The visual representation given above demonstrates that the change in direction for predicted BTI is in accord with the BTI assessed using sensory analysis.

Summary and conclusions

The success of 3D pharmacophore connection techniques with the molecular docking method is largely based on intuitive interpretation and creation of pharmacophore models and precise ligand docking into the molecular mould (MM). Computer analysis confirmed bitter taste stimulation by methylxanthines only in nucleophilic active areas (Nu_1-Nu_2-Nu_3 or Nu_3-Nu_2-Nu_1 system). The study proves that the bitter taste activity of caffeine and other analyzed methylxanthines depends on the symmetric arrangement of nucleophilic active sites in the xanthine skeleton typical of their structure. Consequently, the spatial arrangement of the molecules included in the analysis makes the nucleophilic sites B_1, B_2 and B_3 very easily available for the ligand-receptor protein interactions. However, the structure of the compounds has yet another advantage, namely it facilitates rapid activation of the receptor protein regardless of the fragment of the caffeine structure approaching the protein.

Based on the study reported in this article, an equation was developed to express the intensity of bitter taste (BTI= $\sum$ NPI + $\sum$ NIS). The components of the equation are NPI and

NIS descriptors which represent the number of active sites and the number of possible fittings of the ligand into the area of receptor recognition. The simple equation is useful for predicting the degree of bitter taste of all hypothetical ligands on the sole basis of data contained in the ligand's spatial structure. Therefore, a possible application of results of the study is faster and better control of the organoleptic quality of plant foods, food products, nutraceuticals and drugs.

Acknowledgements

The study was supported by Ministry of Science and Higher Education of Poland in the years 2008-2011 within the project No. NN312 187835.

References

Adler E., Hoon M.A., Mueller K.L., Chandrashekar J., Ryba N.J., Zuker CS., 2000. A novel family of mammalian taste receptors. Cell., 100, 693–702.

Behrens M. et al., 2007. Gustatory expression pattern of the human TAS2R bitter receptor gene family reveals a heterogenous population of bitter responsive taste receptor cells. J Neurosci., 27, 12630–12640.

Biarnés X. et.al., 2010. Insights into the Binding of Phenyltiocarbamide (PTC) Agonist to Its Target Human TAS2R38 Bitter Receptor. *PubMed Central*. Published online 2010 August 25. doi: 10.1371/journal.pone.0012394

Bielenica A., Kossakowski J., 2010. Zastosowanie metod obliczeniowych do wyznaczania budowy modeli farmakoforowych receptorów 5-ht_{1a}, 5-ht_{2a} oraz 5-$ht_{7.}$ Biul. Wydz. Farm. WUM, 1, 1-12; http://biuletynfarmacji.wum.edu.pl/

Caballero J., 2010. 3D-QSAR (CoMFA and CoMSIA) and pharmacophore (GALAHAD) studies on the differential inhibition of aldose reductase by flavonoid compounds Journal of Molecular Graphics and Modelling, Vol. 29, 3, 363–371.

Delwiche J.F., Halpern B.P., Lee M.Y., 1996. A comparison of tip of the tongue and sip and spit screening procedures Food Quality and Preference, Vol. 7, 3–4, 293–297.

Frank O., Blumberg S., Kunert C., Zehentbauer G., Hofmann T., 2007. Structure determination and sensory analysis of bitter-tasting 4-vinylcatechol oligomers and their identification in roasted coffee by means of LC-MS/MS, Journal of Agricultural and Food Chemistry, 55, 1945–1954.

Frank O., Blumberg S., Krümpel G., Hofmann T., 2008. Structure determination of 3-O-caffeoyl-epi--quinide, an orphan bitter lactone in roasted coffee, Journal of Agricultural and Food Chemistry, 56, 9581–9585.

Jasiczak J., Kikut-Ligaj D., 2007. Prediction of the Bitter Taste. Part III. Methodology, [in:] Current Trends in Commodity Science. Ed.: Zieliński R., Publisher: Wydaw. AE, Poznań, Poland, Vol. 2, 855–862.

Kenji M. et al., 2008. Bitter peptides activate hTAS2Rs, the human bitter receptors. Biochem. Biophys. Res. Commun., 365, 851–855.

Kikut-Ligaj D., Jasiczak J., 2004a. The molecular mould of bitter taste receptor in combination with the QSAR method for prediction of taste, [in:] Commodity Science in Global Quality Perspective. Commodity – Trade – Environment, Ed.; Rang W., Changju Y., Min J., Jianghua L., Xuzhe F., Liqin L., Publisher: China Agriculture Press – Beijing, China, Volume 1, 148.

Kikut-Ligaj D., Jasiczak J., 2004b. The application of molecular mould of bitter taste receptor in combination with the QSAR method for prediction of taste. Part II. Annals of the Polish Chemical Society, 3, 675–679.
Kikut-Ligaj D., Skolik A., Przybył A., 2005. Cytisine derivatives as a structural determinant for the bitter molecules, [in:] Current Trends in Commodity Science. Ed.; Zieliński R., Publisher: Wydaw. AE, Poznań, Poland, 2, 1054–1058.
Kikut-Ligaj D., Jasiczak J., 2006. Bitter taste, its prognostication and role in nourishment. Polish Journal of Commodity Science, 1, 15–28.
Kikut-Ligaj D., Skolik A., Włodarczak J., Wysocka W., 2010a. Prediction of bitter taste, of some bis--quinolizidine alkaloids. Polish Journal of Commodity Science, 3, 103–114.
Kikut-Ligaj D., Jasiczak J., Libera J., 2010b. Prediction of bitter taste of flavonoids contained in grapefruit, Zeszyty Naukowe Uniwersytetu Ekonomicznego w Poznaniu, 158, 38–46.
Kim J.H., Odutola J.A., Popham J., Jones L., von Laven S., 2001. Tautomeric energetics of xanthine oxidase substrates: xanthine, 2-oxo-6-methylpurine and lumazine. Journal of Inorganic Biochemistry, 84 (1), 145–150.
Klimacka-Nawrot E., Suchecka W., Błońska-Fajfrowska B., 2007. Przydatność gustometrii swoistej do oceny sprawności zmysłu smaku. Wiadomości lekarskie, 60(9–10), 409–414.
Klimacka-Nawrot E., Suchecka W., 2008. Metody badań wrażliwości smakowej. Wiadomości lekarskie 61(7–9); 207–210.
Kreppenhofer S., Frank O., Hofmann T., 2011. Identification of (furan-2-yl) methylated benzene diols and triols as a novel class of bitter compounds in roasted coffee, Food Chemistry, Vol. 126, 2, 441–449.
Lauria A., Ippolito M., Fazzari M., Tutone, M., Di Blasi F., Mingoia F., Almerico A.M., 2010. IKK--beta inhibitors: An analysis of drug-receptor interaction by using Molecular Docking and pharmacophore 3D-QSAR approaches. J. Mol. Graph. Model., 24, 29(1), 72–81, 22.
Martin Y.C., 2007. Pharmacophore Modeling: 1–Methods. Comprehensive Medicinal Chemistry II, 4.06,119–147.
Miguet L. et al., 2006. Computational studies of ligand-receptor interactions in bitter taste receptors. J. Recept. Signal Transduct. Res., 26, 611–630.
Nehlig A., Daval J-L., Debry G., 1992. Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. Brain Research Reviews, 17, 2, 139–170.
Nowotna-Kromolicka N., Kikut-Ligaj D., Jasiczak J., 2007. Preliminary model of the Multipoint Molecular Mould of Bitter Taste Receptor, [in:] Current Trends in Commodity Science, Ed. Zieliński R., Publisher: Wydaw. AE, Poznań, Poland, Vol. 2, 928–931.
Nunes S.C.C., Jesus Lopes A.J., Ermelinda M., Eusébio S., Redinha J.S., 2008. Conformational preferences of 2-isopropylaminoethanol in aqueous solution using the CPCM continuum solvation model Journal of Molecular Structure: Theochem, Vol. 867, Issues 1–3, 30 October Pages 101–106.
Polish standard PN-ISO 8586-1(2), 1998. General guidance for the selection, training and monitoring of assessor – part 1: selected assessor (in Polish).
Polish standard PN-ISO 8589, 1998. General guidance for the design of test rooms (in Polish).
Polish standard PN-ISO 6658, 1998. Sensory analysis-methodology-general guidance (in Polish).
Polish standard PN-ISO 3972, 1998. Sensory analysis-methodology-method of investigating sensitivity of taste (in Polish).
Reed D.R., Tanaka T., McDaniel A.H., 2006. Diverse tastes: Genetics of sweet and bitter perception. Phisiology & Behavior, 88, 215–226.
Seidel T., Ibis G., Bendix F., Wolber G., 2010. Strategies for 3D pharmacophore-based virtual screening. Review Drug Discovery Today: Technologies, Vol.7, No. 4, 221–228.
Shi P., Zhang J., 2006. Contrasting modes of evolution between vertebrate sweet/umami receptor genes and bitter receptor genes. Mol Biol Evol., 23, 292–300.

Stark T., Bareuther S., Hofmann T., 2005. Sensory-guided decomposition of roasted cocoa nibs (Theobroma cacao) and structure determination of taste-active polyphenols, Journal of Agricultural and Food Chemistry, 53, 5407–5418.

Stevens D.A. et al., 1988. Explaining individual differences in flavor perception, [in:] Food Acceptability, ed. Thompson D.M.H., Elsevier Temussi P.A., 2009. Sweet, bitter and umami receptors: a complex relationship. Trends in Biochemical Sciences, Cell Press, 34, 6, 296–301.

Takano Y., Houk K.N., 2005. Benchmarking the Conductor-like Polarizable Continuum Model (CPCM) for Aqueous Solvation Free Energies of Neutral and Ionic Organic Molecules. J. Chem. Theory Comput., 1 (1), 70–77.

Wolber G., Seidel T., Bendix F., Langer T., 2008. Molecule-pharmacophore superpositioning and pattern matching in computational drug design. Drug Discovery Today, Vol. 13, 1–2, 23–29.

Zhan Chen, Baker N.A., Wei G.W., 2010. Differential geometry based solvation model I: Eulerian formulation Journal of Computational Physics, Vol. 229, 22, 8231–8258.

9

TASTE EVALUATION OF SELECTED QUINOLIZIDINE ALKALOIDS

Introduction

Alkaloids are one of the largest group of natural compounds, mainly plant-derived, very seldom present in animal organisms and insects and arthropods as pheromones with scaring, protecting and alarming functions [Numata 1987]. Alkaloids occupy a special position among active substances present in spices, and particularly in stimulants. Currently, about 10,000 alkaloids are know, out of which the structure has been determined in 8,000. Many of them act as strong narcotics and poisons, but some in small doses are used as soporifics and painkillers.

The first alkaloid was discovered by a German pharmacist Sertűngerg in 1806, isolating morphine from opium. This was a sensational discovery and soon search began for similar substances in other medicinal materials [Pelletier 1970]. The name alkaloid, meaning "alkali-like", was proposed by a pharmacist W. Meissner in 1819 for organic compounds of natural origin with one or more nitrogen atoms in the molecule.

Alkaloids are alkaline substances, containing at least one nitrogen atom (in heterocyclic ring in most cases). Structure of alkaloids is complicated, diversified and in many cases not fully determined. Most often, they are crystalline compounds, hardly soluble in water, with bitter taste, optically active, isolated from plants by extraction and distillation with vapour. Pure compounds are obtained by crystallization, chromatography, counter-current extraction or in ion exchangers. Alkaloids most frequently occurs in groups, and some plant species have a few dozen various, often related compounds of that type. So far, their presence has been confirmed in over 1,200 plant species belonging to about 100 families, but the numbers are still growing. Many plants with high contents of alkaloids are poisonous, but with suitable dosing they play an important role in medicine.

Growing interest in quinolizidine alkaloids have been observed in recent years due to their large practical and synthetic use. These alkaloids are used in synthesis of many organic compounds, and their biological activity is particularly interesting.

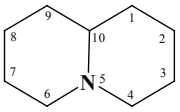
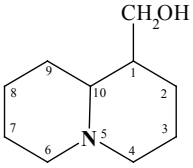
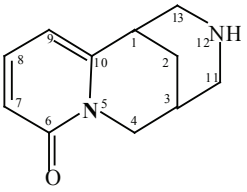
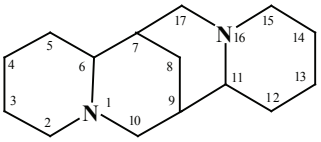
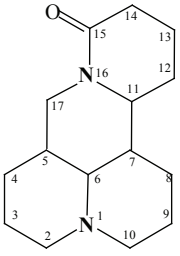
Due to basic molecule structure, the quinolizidine alkaloids can be divided into the following groups: [Jasiewicz 2003, Wysocka 1996] (Tab. 1)

1. compounds which skeleton comprises one quinolizidine system, the main representative of that group is lupine,

2. compounds which skeleton comprises a quinolizidine system condensed with one piperidine system in the 1-3 position, the main representative of that group is cytisine,
3. compounds which skeleton comprises two quinolizidine systems condensed with each other in the 1-3 and 1`-3 positions, the main representative of that group is sparteine,
4. compounds which skeleton also comprises two quinolizidine systems, but connected in the 1-3 and 1`-9` positions, the main representative of that group is matrine.

Table 1

Groups of quinolizidine alkaloids

No group	Compound name	Structural formula
I	quinolizidine	
I	lupinine	
II	cytisine	
III	sparteine	
IV	matryne	

This paper deals with alkaloids from group II, with cytisine as the main representative.

This compound is obtained on industrial scale from the seeds (content 1–3%), sometimes leaves (0.3%) and flowers (0.2%) of golden chain tree (*Laburnum anagyroide)*.

Due to similar action to that of nicotine, cytisine is used in small doses to treat the symptoms of nicotine craving. It is sols in tables under tradename Tabex. Its huge advantage is that it does not lead to addiction [Skolik et al. 2006, Kikut-Ligaj et al. 2006]. Within the studies of chemoreception of the bitter flavour, it was decided to prepare a series of sulphur derivatives of cytisine – cytisin tio-analogues and determine their flavour by means of the sense of taste used as a measuring instrument, keeping adequate evaluation conditions and requirements in terms of evaluating people and methods adequate to the tasks.

Full explanation of the selection of sensory analysis to test the bitter flavour of selected cytisin tio-analogues involved an effective, total identification of their possibilities and focused on the scaling methods. Familiarity with them allowed to avoid evaluation errors and to maintain accuracy and result repeatability as high as possible [Skolik 2006, Skolik 2007a, Skolik 2011].

After comparison of chosen scaling methods, it was fund out that the most accurate scale to determine the flavour of cytisin tio-analogues is a hybrid of linear and category scales [Skolik 2007b].

Materials and Methods

Tests of the cytisine tioanalogues flavour was carried out in accordance with Polish Standards.

In order to determine the sensory sensitivity of candidates for evaluators, the tests were made for:

- "taste Daltonism", which is the ability to identify correctly four basic tastes – to eliminate the people who are incapable of distinguishing four basic tastes correctly,
- taste sensitivity thresholds. Not all stimuli can cause a sensation; for the sensation to occur, the stimulus must reach a sensitivity for a given individual in the area of a given sense. This is threshold value or a sensitivity threshold for a given sense. The threshold is used in sensory analysis as one of the measures for individual sensitization of the people who are to carry out the evaluation,
- difference thresholds allowing to determine the increase of stimulus intensity which causes a perceptible increase of intensity.

A group of 20 people was selected for the study, out of whom the group of ten "evaluators" was selected die to their particular ability in organoleptic evaluation. This group formed a so-called "sensory panel" of people with good repeatability of evaluations and taste memory. This proven panel performed the sensory evaluation of cytisine tioanalogues in terms of intensity of the bitter favour.

Sensory tests involved use of alkaloids with the same concentration (0.0025 ml/l), which were suitably coded (Tab. 2). Quinine hydrochloride was used as a bitter taste standard.

Scaling method was used to evaluate the flavour. The method involved evaluation of bitterness of tested samples on a hybrid linear-category scale. The tests included evaluation of alkaloids samples and the samples containing distilled water and quinine hydrochloride (standard of the bitter taste). (Fig. 2).

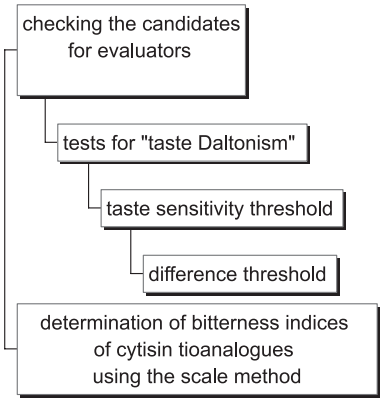


Fig. 1. Research methodology

Statistical calculations were performed using the *STATISTICA 9.0*. software package. The presented results re a mean from two repetitions. The condition of normal distribution was met for all studies (Shapiro-Wilk test), so was the equality of variances condition (Levene test). A single-factor analysis of variance (ANOVA) was performed. The Tukey's test was performed to verify significance of differences between the mean values. $\alpha = 0.05$ was used a critical significance level.

DETERMINATION OF THE BITTERNESS LEVEL USING THE SCALING METHOD

Name:…… Date: …….

Task: evaluate the perceived bitterness of samples 0-5 and write the code according to your perception.

Perceived bitterness	none	very weak	weak	moderate	strong	vary strong
% bitterness	0%	20%	40%	60%	80%	100%
Sample code						

Fig. 2. Bitterness level evaluation sheet

Table 2

Alkaloid codes

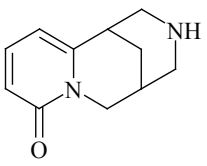
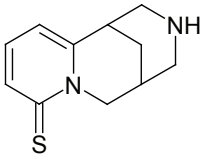
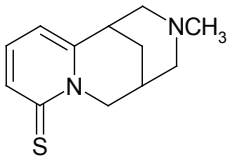
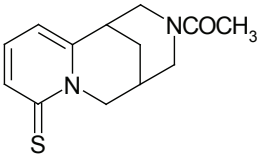
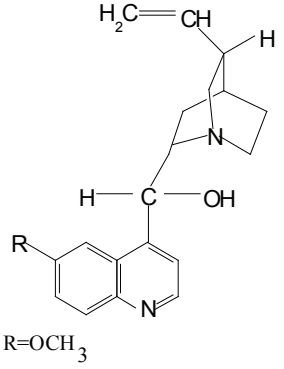
Code	Compound name	Structural formula
0	Woda	solvent
1	cytisine	

Table 2 cont.

2	2-tiocytisine	
3	N-metylo 2-tiocytisine	
4	N-acetylo 2-tiocytisine	
5	Quinine (standard)	

Results and Discussion

The evaluators evaluated the coded samples on the scale from unperceived bitterness (0%) to vary strong bitterness (100%).

After grouping and averaging, the results were systematized using suitable statistical methods (Tab. 3).

Table 3

Percent alkaloids bitterness index

COMPOUND	BITTERNESS INDEX OF SAMPLE	
	LINEAR SCALE	CATEGORY SCALE
water	1.0 ± 2.1	not bitter
N-methyl-2-tiocytisine	60 ± 2.2	moderately bitter

Table 3 cont.

N-acetyl 2- tiocytisine	50± 2.2	moderately bitter
2- tiocytisine	80± 2.3	strongly bitter
cytisine	80± 2.3	strongly bitter
quinine	99.0 ± 2.1	very bitter

Conclusions

The problem of testing quinolzidine alkaloids, particularly in terms of biological activity, is still relevant, and search for new testing techniques create wide possibilities of a deeper understanding and use of these interesting compounds.

The results showing the graphical scale of the bitter flavour of cytisine derivatives were used to proposes a scaling method and a tool to study and visualize the taste perception and allowed a new systematics of these compounds according to their taste.

Cytisine and 2- tiocytisine were the most bitter alkaloids, with the 80% index, and N-acetyl 2- tiocytisine had the lowest bitterness index of 50%.

The results have indicated which selected cytisine tioanalogues will be used to shape the molecular matrix of the bitter taste receptor and allow their use in further studies of the bitter taste chemoreception [Jasiczak et al. 1999, Skolik A. et al. 2002].

References

Baryłko-Piekielna N., Matuszewska I., 2009. Sensoryczne badanie żywności. WN PTTŻ, Kraków, 165.

Jasiczak J., Wysocka W., Skolik A., 1999. Matryca molekularna receptorów smaku gorzkiego w badaniach struktury alkaloidów chinolizydynowych. w: Na pograniczu chemii i biologii. vol. III, publisher: Wyd. Naukowe UAM, 503–529.

Jasiewicz B., Boczoń W., 2003. Alkaloidy bis-chinolizydynowe – struktura i właściwości, Na pograniczu Chemii i Biologii, Wyd. Uniwersytet im Adama Mickiewicz w Poznaniu, red. Koroniak H., Barciszewski J., 199–203.

Kikut-Ligaj D., Skolik A., Przybył A., 2006. Cytisine derivatives as tools for modeling molecular mould of the bitter taste.3rd Central European Conference Chemistry Towards Biology, Uniwersytet Jagielloński w Krakowie, 31.

Numata A., Ibuka T., 1987. The Alkaloids, ed. A. Brossi, Academic Press, New York, vol. 31, 193–307.

Pelletier S.W., 1970. Chemistry of the alkaloids, ed. S.W. Pelletier, Van Nostrand Reinhold Company, New York, 1–9.

PN-ISO 5492, 1997. Sensory analysis – vocabulaire.

PN-ISO 8586-1, 1998. General guidance for the selection, training and monitoring of assessor part 1: selected assessor.

PN-ISO 8586-2, 1998. General guidance for the selection, training and monitoring of assessor part 2: experts.

PN-ISO 8589, 1998. General guidance for the design of test rooms.

PN-ISO 6658, 1998. Sensory analysis-methodology-general guidance.

PN-ISO 3972, 1998. Sensory analysis-methodology-method of investigating sensitivity of taste.

PN-ISO 4121, 1996. Sensory analysis-methodology-evaluation of food products by methods using scales.
Rejewski M., 1992, Rośliny przyprawowe i używki roślinne, Państwowe Wydawnictwo Rolnicze i Leśne, 28.
Skolik A., 2011. Smak w analizie sensorycznej, Wyd. Uniwersytet Ekonomiczny w Poznaniu, 62–64.
Skolik A., 2007a. Use of scaling methods in investigating of the bitter taste, Current Trends in Commodity Science, 9th International Comm0dity Science Conference (IGWT) Poznań, vol. II, 999–1003.
Skolik A., 2007b. Selected sensory scaling methods in product Quality assurance system, III International Conference on Quality and Safety in Food Production Chain, Wrocław University of Enviromental and Life Sciences, Faculty of Food Department of Animal Products Technology and Quality Management, Polish Scienty of Food Technologists, Wrocław, 384–385.
Skolik A., Przybył A., Kikut-Ligaj D., 2006. The scaling method as a tool for visualization of the bitter taste. 15th IGWT Symposium Global Safety of Commodity and Environment. Quality of Life. red. Pugachevsky G.F., Pirtulskaya N.V., Rudavskaya H.B. Kijów: Kyiv National University of Trade Economics, 871–874
Skolik A., 2006. Use of methods of sensory analysis in investigatting the bitter taste of selected alkaloids, III International Conference, Role of Science of Commodities in Quality Management in Knowledge – Based Economy, Innovativeness in Product and service Quality, Development, Technical University of Radom, Poland, red. J. Żuchowski, (ISBN 83-7351-167-9), 61–62.
Skolik A., Jasiczak J., Kikut-Ligaj D., 2002. Shape of bitter receptors studied by computer modeling, Poznań 7th International Commodity Science Conference, 640–645.
Wysocka W., Brukwicki T., 1996. Conformational equilibria in quinolizidine alkaloids. J. Mol. Struct., 385, 23–33.
Wysocka W., Przybylak J.K., 2003. Synteza i struktura tioanalogów cytyzyny. XLVI Zjazd PTCh i SITPCh, 1214

10

COMPARISON OF ANALYTICAL METHODS OF PHENOLIC COMPOUNDS DETERMINED IN CEREAL GRAINS

Introduction

Wheat is a major crop and an important component of the human diet. There are several reports on the content of phenolics in different varieties of wheat grains or their different parts (Tab. 1). Increasing interest in phenolic compounds originates mainly from their antioxidant properties [Adom and Liu 2002, Graf 2002]. Particular attention has been paid to their role as "free radical scavengers" and has provoked numerous studies into phenolic compounds in many plants, including cereals. Cereals contain a wide range of phenolic compounds, of different chemical structures, of which phenolic acids are of significance. In wheat grains, ferulic acid occurs in the highest amounts [Kampa et al. 2004, Kikuzaki et al. 2002]. Phenolic acids contribute not only to the sensory properties of cereal products but also to their health benefits such as anticancer or antidiabetes properties. It has been found that phenolic content in cereal products correlate positively with their antioxidant activity [Adom et al. 2003, 2002, Grassseli and Bulkin 1991]. Due to the content of phenolic compounds, including ferulic acid, and their biological activity cereal products may constitute an important part of human daily diet [Lachman et al. 2003, Liyana-Pathirana and Sahidi F. 2005]. Phenolic compounds have also appear to play a role in the resistance of the cultivar of wheat to *Fusarium culmorum* [Akin 1995]. Several analytical methodologies have been reported for the qualitative and quantitative determination of phenolic compounds in various plant extracts [Andreasen et al. 2000, Cadahia et al. 1997, Carrasco-Pancrobo et al. 2007, Cavaliere et al. 2005, Li et al. 2005].

The objective of the present research was to compare also various methods used for preparing and/or treating samples to determine the phenolic content in c.

Determination of total phenolic and flavonoid contents methods

The need for profiling and identifying individual phenolic compounds has seen traditional methods replaced by high-performance chromatographic analyses. Various separation techniques (HPLC, GC, CE), coupled with mass spectrometry (HPLC–MS, GC–MS,

CE–MS) [Donna et all. 2007, Dowell 1997, Dykes and Rooney 2007, Hagerman et al. 1998] or nuclear magnetic resonance (NMR) [Harborne et al. 2000, 1986, Hosseinian et al. 2008, McCallum et al. 1989, Meyer et al. 2000], have been found as valuable tools for the characterization of polyphenols content in wheat samples. The limited volatility of many phenols, the instability of derivatized phenolic compounds and the potential for further chemical modification of the dimers during derivatization have restricted the application of GC to their separation [Mazza et al. 2005, Peñalvo et al. 2005, Saulnier et al. 2007]. Nevertheless, HPLC currently represents the most popular and reliable technique for analysis of phenols [Rice-Evans et al. 1997, Singleton and Rossi 1965]. In recent years, liquid chromatography/time-of-flight mass spectrometry (LC/TOF-MS) has been used to separate and characterize secondary metabolites of some complicated components such as wine antioxidants [Yu et al. 2000], olive phenolic compounds [Zhang et al. 2008], olive secoiridoids [Chen et al. 2007], anthocyanins [Feng et al. 1988a] and tectorigenin [Ferrer et al. 2005, Fu et al. 2009]. Benefiting from the increased power of high resolution, accurate mass measurements, LC/TOF-MS provides the elemental compositions of unknown peaks with high accuracy (routinely below 10 ppm) in complex matrices [Fulcher and Wong 1980, Faulds et al. 2003, Kim et al. 2006a]. It has become a competitive technology for the accurate and sensitive characterization of some complicated components in complex matrices [Kasum et al. 2002, Kim et al. 2006b].

Even though the absolute numbers differed, a reasonably good correlations were obtained when comparing total phenolics determined by the most widely used Folin-Ciocalteau method with the RP-HPLC and the cyclic voltammetry methods. The correlation coefficient was better for the RP-HPLC than for the cyclic voltammetry [Kim et al. 2006b, Lempereur et al. 1997, Rao and Muralikrishana 2004]. The Folin-Ciocalteu method has the advantages of being relatively simple and fast method for phenolic quantitative determinations which basis is well understood. However, since its poor specificity the results could give high variations. Moreover, using Folin-Ciocalteu method or cyclic voltammetry only total phenolics could be determined in the samples without qualitative determination. To get better insight into the phenolic profiles liquid chromatography methods are used [Peterson 2001]. HPLC combined with various detectors like PDA, UV, fluorescence detector enables to isolate, identify and determine individual phenolics in the grain products [Orśak et al. 2001]. When coupled with mass spectrometry the determination was often made more effective. The disadvantage of the chromatographic methods could be the process of sample preparation which is often time consuming and it requires experience from the analyst. The sample preparation is of great importance in order to diminish the negative effect of the matrix. To this end different techniques are used, such as solid-phase extraction (SPE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) [Hvattum 2002, Izydorczyk and Billierderis 1995, Peterson et al. 2002]. When choosing the technique of sample preparation not only the efficiency of extraction is taking into account but also the reduction of the solvents used as well as time and cost of the procedure.

Other phenolic compounds in wheat bran include apigenin and other flavonoids [Run-Cang et al. 2005]. Miyamoto and Everson [1958] identified catechin and catechin tannin as the precursors of brown pigment and showed a correlation between kernel color. McCallum and Walker [1990] [Dowell 1997, McCallum and Walker 1990] suggested that trace levels of proanthocyanidins in the bran contributed to seed coat color in wheat. Wheat also contains a major group of 5-n-alkylresorcinols [Bunzel et al. 2001, Hernanz et al. 2001, Liu et al. 2004a] with odd-number side chains of C15-C25, 5-(2-oxoalkyl), and 5-(2-oxoalkenyl) resorcinols

[Carter and Edwards 2001]. NIR spectroscopy has been used to distinguish red from white wheat before NaOH soak [Baranska et al. 1987, Carter and Edwards 2001, Hatcher and Kruger 1997] and after NaOH soak [Baeten et al. 1998, Inglet and Munck 1973, Jacobs et al. 1998]. However, identification of individual components is difficult and many times impossible. Aromatic compounds, particularly the cinnamic acid derivatives, exhibit characteristic and intense Raman spectra. Generally, the intensity of Raman bands for nonpolar or slightly polar groups is higher than for polar groups, and intensity of stretching vibrations is higher than that for deformation vibrations. Raman intensity is also usually higher for symmetric vibrations than for antisymmetric vibrations and enhanced for stretching vibrations of multiple bonds, such as that of C=C [Ma and Philips 2002]. Raman spectroscopy has been applied to determine protein and apparent amylose contents of milled rice [Lewis and Edwards 2001, Joven et al. 2008], amylose content in maize [Kivilompolo et al. 2008, Himmelsbach and Ankin 1998], benzyl and acetyl modification of starches [Himmelsbach et al. 2001], and for monitoring a bioprocess for ethanol production [Garcia-Villaba et al., in press, Peyron et al. 2002, Philips et al. 2001, Philips et al. 1999a]. Ma and Phillips [2002] have reviewed the applications of FT-Raman spectroscopy to cereal science. Wheat sections have been studied by confocal Raman microspectroscopy, and the spatial distribution of protein and phenolic compounds has been described [Martinez--Vaverde et al. 2002, Phillips et al. 199b, Sivakesawa et al. 2001].

Occurrence of phenolic acids in cereal grains

Weidner et al. [2000] analyzed the content of phenolic acids in kernels of Polish varieties of wheat, rye, and triticale. The characteristics covered three fractions: free phenolic acids, acids released from soluble ester bonds, and acids released from soluble glycosides. The results of literature studies clearly indicate a higher content of ferulic acid in grain depending on the variety (Elena, Alba variety), especially when determined in ester or glycoside bonds (Tab. 1). Besides ferulic acid, sinapic, p-coumaric, and caffeic acids were pre-

Table 1

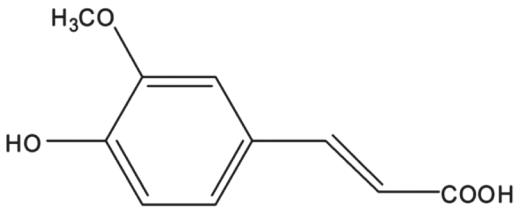
Phenolic acids reported in cereal grains (26)

Phenolic acid	Grain	Reference
Gallic	Rice, sorgum	(5, 20, 53, 68, 85)
Protocatechuic	Barley, maize, oat, rice, rye, sorgum, wheat	(9, 63, 114, 134)
p-Hydroxybenzoic	Barley, maize, oat, rice, rye, sorgum, wheat	(21, 128, 134)
Gentisic	Sorgum	(50, 62, 90)
Salicylic	Sorgum, barley, wheat	(81, 82, 98)
Vanilic	Barley, maize, oat, rice, rye, sorgum, wheat	(110, 112, 115)
Syringic	Barley, maize, oat, rice, rye, sorgum, wheat	(6, 7, 12, 73, 133)
Ferulic	Barley, maize, oat, rice, rye, sorgum, wheat	(22, 37, 72)
Cafeic	Maize, oat, rice, rye, sorgum, wheat	(6, 7, 12, 73, 133)
o-Coumaric	Barley	(74, 75)
m-Coumaric	Barley	(74, 75, 83)
p-Coumaric	Barley, maze, oat, rice, rye, sorgum, wheat	(74, 75, 83, 84, 111)
Cinnamic	Sorgum, wheat	(96, 103)
Sinapic	Barley, oat, rice, rye, sorgum, wheat	(6, 74, 84, 103, 113)

sent in all the analyzed phenolic fractions in wheat, rye, and triticale. Papers by Feng et al. [1989b] also support the thesis that the following acids occur in the highest amounts in rye: ferulic (1079 μg/g d.m.), sinapic (76 μg/g d.m.) and p-coumaric (35 μg/g d.m.). Chen et al. [2004] analyzed the content of phenolic acids in five cereal varieties: wheat, barley, rye, oat and buckwheat. The following acids occurring in a free or ester-bonded form were identified in the analyzed grains: vanillic, syringic, ferulic, and coumaric. Rye, oat, and buckwheat grain also contained sinapic and caffeic acids. Ferulic acid predominated among the phenolic acids found in the analyzed varieties of wheat and barley while syringic acid predominated in the grain of oat, rye, and buckwheat. The total amount of phenolic acids occurring in both forms was the highest in rye and oat.

Ferulic acid in wheat grain

Studies into the occurrence of phenolic acids in cereal grains support the thesis of the distinguished content of ferulic acid (Fig. 1) among the phenolic compounds of wheat grain [Peyron et al. 2001]. This results from, among others, the fact that ferulic acid (3-methoxy-4-hydroxycinnamic acid) is the major phenolic acid occurring in the cell walls of monocotyledones [Piot et al. 2000, Pussayanawin et al. 1988, Smigel 1992, Zhinsen et al. 1999] The following acids are its precursors: p-coumaric (p-hydroxycinnamic) and caffeic (3,4-dihydroxycinnamic). They are synthesized in plants from phenylalanine and L-tyrosine on the shikimic pathway [Yao et al. 2004]. Ferulic acid appears in the form of *cis*- and *trans*-isomers [Sun et al. 1996]. Molecules of ferulic acid may bind oxygen atoms forming dimers [Soine 1964]. Numerous dehydrodimers of ferulic acid, including 5-5_-diferulic acid ((E,E)-4,4_-dihydroxy-5,5_-dimethoxy-3,3_-bicinnamic) occurring in the largest amounts in cereal grain were found in the pentosane fraction of wheat [Ram et al. 2002, Yu et al. 2001]. The blue autofluorescence of ferulic acid confirms its presence in the cell walls of the wheat kernel aleurone layer [Thompson 1994]. Ferulic acid esters reveal blue fluorescence at pH 5.8 and change their color into green at pH 10 [Rybka et al. 1993]. Fulcher et al. [1980] noted that the blue-white autofluorescence of wheat kernel cell walls is exactly the same as that revealed by pure crystals of ferulic acid. It indicates that ferulic acid, whose concentration in the aleurone layer is very high, may be responsible for the fluorescence of cell walls. It was supported by the studies of Scalbert et al. [1985]. Rybka et al. [1993] found that the ferulic acid content in wheat kernels in all its forms amounts to 31.0 μg/g. On the other hand, Pussnawin et al., [1988] determined the total ferulic acid content at the level of 500 μg/g. Rybka et al. [1993] confirmed the predominating character of this acid among free phenolic acids and those in ester bonds. Lachman et al. (2003) showed a high genetic (variety-dependent) diversity of the ferulic acid content in durum wheat grains (from 0.693 to 2.443 mg/g d.m.). The mean content of that acid was higher compared to that reported for *Triticum aestivum*. Hatcher and Kruger [1997] analyzed the content of phenolic acids, including ferulic acid, in grains and flour of different purity obtained from five quality classes of Canadian wheat: CWSWS (CanadianWestern Soft White Spring), CPSR (Canadian Prairie Spring Red), CWRW (Canadian Western Red Winter), CWRS (Canadian Western Red Spring), and CWES (Canadian Western Extra Strong). They isolated three fractions of phenolic acids: free phenolic acids,



(*E*)-3-(4-hydroxy-3-methoxyphenyl)acrylic acid
(ferulic acid)

Fig. 1. Chemical structure of ferulic acid

acids bound into insoluble complexes, and acids occurring in soluble ester linkages. The highest share (80%) in the total sum of phenolic acids was reported for acids occurring in insoluble complexes. Of all the acids in this fraction, only ferulic acid could be determined quantitatively; its content in the whole kernels ranged from 274.8 to 337.6 μg/g, depending on the quality class of wheat. The lowest content was found in CWRW and CWES class grain. These classes represent the highest quality. This suggests a relation between high technological quality of wheat kernels and low content of ferulic acid in insoluble linkages. The content of ferulic acid determined in the soluble ester linkages of wheat grains reached 8.6–17.6 μg/g, while in the fraction of free phenolic acids–from 0.2 to 6.3 μg/g, depending on the wheat variety.

Phenolic acid in oat grain

Phenolic compounds present in oats may contribute to functional and nutritional properties of the grain. It is well known that phenolic acids, which are abundant in whole grains, have antioxidant characteristics [Onyeneho and Hettiararchy 1992] They function in the body as free-radical scavengers, complexers of prooxidant metals, reducing agents, and quenchers of singlet-oxygen formation [Sancho et al. 2001]. Antioxidants protect the body from degenerative diseases such as cancers [Velioglu et al. 1998] and heart disease. Hydroxycinnamates such as ferulic acid, caffeic acid, and p-coumaric acid reduce low-density lipoprotein oxidation [Meyer et al. 2000, Waldron and Parr 1996], potentially protecting the body from atherosclerosis [Graselli and Bulkin 1991, Wood et al. 2005] In addition to the potential health effects, phenolic compounds affect the flavor of processed oat products [Waldron and Parr 19, Wu et al. 1999]. Phenolic compounds with antioxidant activity have been identified in oats [Zhisen et al. 1999, Zielinski and Kozlowska 2000]), but quantitative data are highly variable and difficult to compare due to differences in methods of extraction and analysis [Harborne and Williams 2000, Onyeneho and Hettiararchy 1992]. The effects of processing on the content and activity of potential antioxidative compounds in oats are even less well characterized [Hernandez et al. 2001, Rao and Muralikrisna 2004]. The phenolic acids are asymmetrically distributed within the grain [Liu et al. 2004b], and some, such as ferulic acid and p-coumaric acid, occur mostly bound to cell walls [Peyron et al. 2001].

Concluding remarks

There are a variety of analytical methods for phenolic determination in cereal grains which results in a great discrepancies of the final results comparison. Thus, furher detailed research are needed.

Present knowledge of phenolic compounds in cereal grain, particularly wheat grain, indicates a series of correlations. The most important is the variety-dependence of phenolic compound content, however, the variability of this trait for different varieties affected by biotic and abiotic factors has not yet been explained. On the other hand, it was found that the content of ferulic acid is many times greater in the seed coat of wheat and its content in ester linkages is many times greater than in insoluble complexes. These findings may be a starting point for further studies into the application of ferulic acid content as a discriminate of ash content in flour. Moreover, provided quick methods based on fluorescence measurement can be developed, they can serve as another milling quality technique to be applied in mills.

References

Adom K.K., Liu R.H., 2002. Antioxidant activity of grains. Journal of Agriculture and Food Chemistry, 50, 6182–6187.

Adom K.K., Sorrells M.E. , Liu R.H., 2003. Agric J., Food Chem., 51, 7825.

Adom K.K., Liu R.H., 2002. Agric J., Food Chem., 50, 6182.

Akin, D.E., 1995. Microspectrophotometric characterization of aromatic constituents in cell walls of hard and soft wheats. J. Sci. Food. Agric., 68, 207–214.

Al-Ruqaie I., Lorenz K., 1992. Alkylresorcinols in extruded cereal brans. Cereal Chem., 69, 472–475.

Andreasen M.F., Christensen L.P., Meyer A.S, Hansen A., 2000b. Ferulic acid dehydrodimers in rye (Secale cereale L.). Journal of Cereal Science, 31, 303–307.

Andreasen M.F., Christensen L.P., Meyer A.S., Hansen A., 2000a. Content of phenolic acids and ferulic acid dehydrodimers in 17 rye (Secale cereale L.) varietes. Journal of Agriculture and Food Chemistry, 48, 2837–2842.

Andreasen M.F., Christensen L.P., Meyer A.S., Hansen A., 2000. J. Agric. Food Chem., 48, 2837.

Andreasen M.F., Kroon P.A., Williamson G., Garcia-Conesa M.T., 2001. Intestinal release and uptake of phenolic antioxidant diferulic acids. Free Radical Biology & Medicine, 31, 304–314.

Baeten V., Hourant P., Morales M.T., Aparicio R., 1998. Oil and fat classification by FT-Raman spectrscopy. J. Agric Food Chem., 46, 2638–2646.

Baranska H., Labudzinska A., Terpinski J., 1987. Laser Raman Spectrometry: Analytical Applications. Ellis Horwood: Chichester, UK.

Benzecri J.P., 1973. L'analyse des données, Tome 2: L'Analyse des Correspondances, Dunod, Paris.

Bunzel M., Ralph J., 2001. Diferulates as structural components in soluble and insoluble cereal dietary fibre. Journal of Agriculture and Food Chemistry, 81, 653–660.

Cadahía E., Conde E., García-Vallejo M.C., Fernandez de Simon B., 1997. Phytochem. Anal., 8, 78.

Carrasco-Pancorbo A., C. Neusu˝_,M. Pelzing, A. Segura-Carretero, A. Fernandez-Gutierrez, 2007. Electrophoresis, 28, 806.

Carter, E.A., Edwards, H.G.M., 2001. Biological applications of Raman spectroscopy, [in:] Infrared and Raman Spectroscopy of Biological Materials. H.-U. Gremlich and B. Yan, eds. Prac. Spectrosc., 24, 421–475.

Cavaliere C., Foglia P., Pastorini E., Samperi R., Laganŕ A., 2005. Rapid Commun Mass Spectrom., 19, 3143.
Chen C.Y., Milbury P.E., Collins F.W., Blumberg J.B., 2007. Avenanthramides are bioavailable and have antioxidant activity in humans after acute consumption of an enriched mixture from oats. Journal of Nutrition, 137, 1375–1382.
Chen C.Y., Milbury P.E., Kwak H.K., Collins F.W., Samuel P., Blumberg J.B., 2004. Avenanthramides and phenolic acids from oats are bioavailable and act synergistically with vitamin C to enhance hamster and human LDL resistance to oxidation. journal of Nutrition, 134, 1459–1466.
Collins F.W., D'Attilio R.Z., 1996. Phenolics of preprocessed wheat: Isolation, structure, and quantitative analysis of major bran constituents. Cereal Foods World, 41,586.
Dimberg L.H., Gissén C., Nilsson J., 2005. Phenolic compounds in oat grains (avena sativa L.) grown in conventional and organic systems. Ambio, 34, 331–337.
Dinelli G., Marotti I., Bosi S., Benedettelli S., Ghiselli L., 2007. S Cortacero-Ramirez Carrasco-Pancorbo A. Segua-Carretero A. Fernandez-Gutierrez A., Electrophoresis, 28, 4212.
Donna L.D., Mazzotti F., Napoli A., Salerno R., Sajjad A., Sindona G., 2007. Rapid Commun. Mass Spectrom., 21, 273.
Dowell F.E., 1997. Effect of NaOH on visible wavelength spectra of single wheat kernels and color-classification efficiency. Cereal Chem., 74, 617–620.
Dowell F.E., 1998. Automated color classification of single kernels using visible and near-infrared reflectance. Cereal Chem., 75, 142–144.
Dykes L., Rooney L.W., 2007. Cereal Foods World, 52, 105.
Escarpa A., Gonzalez M.C., 1999. J. Chromatogr. A 830, 301.
Faulds C.B., Zanichelli D., Crepin V.F., Connerton I.F., Juge N., Bhat M.K., Waldron K.W., 2003. Specificity of feruloyl esterases for water-extractable and water-unextractable feruloylated polysaccharides: influence of xylanase. journal of Cereal Science, 38, 281–288.
Feng Y., McDonald C.E., Vick B.A., 1988a. Cereal Chem., 65, 452.
Feng Y., Mc Donald C.E., Vick B.A., 1988b. C-Glycosylflavones from hard red spring wheat bran. Cereal Chem., 65, 452–456.
Feng Y., Mc Donald C.E., 1989. Comparison of flavonoids in bran of four classes of wheat. Cereal Chem., 66, 516–518.
Ferrer I., Garcıa-Reyes J.F., Mezcua M., Thurman E.M., Fernandez-Alba A.R., 2005. J. Chromatogr. A 1082, 81.
Fu S., Arraez-Roman D., Menendez J.A., Segura-Carretero A., Fernandez-Gutierrez A., 2009. Rapid Commun. Mass Spectrom., 23, 51.
Fulcher R.G., Wong S.I., 1980. Inside cereals–A fluorescence microchemical view, [in:] Cereals for Foods and Beverages, 1–26.
Inglett G.E., Munck L., eds. Academic Press: New York. Geissmann, T., and Neukom, H., 1973. A note on ferulic acid as a constituent of the water-insoluble pentosans of wheat flour. Cereal Chem., 50, 414–416.
García-Villalba R., Carrasco-Pancorboa A., Oliveras-Ferraros C., Vázquez-Martín A., Menéndez J.A., Segura-Carretero A., Fernández-Gutiérrez A., J. Pharm. Biomed. Anal., in press. doi:10.1016/j.jpba.2009.06.021.
Glitso L.V., Knudsen K.E.B., 1999. Milling of whole grain rye to obtain fractions with different dietary fibre characteristics. journal of Cereal Science, 29, 89–97.
Graf E., 1992. Antioxidant potential of ferulic acid. Free Radical Biology & Medicine, 13, 435–448.
Grasseli J.G., Bulkin B.J., 1991. Analytical Raman Spectroscopy. Wiley: New York.
Hagerman A.E., Riedl K.M., Jones G.A., Sovik K.N., Ritchard N.T., Hartzfeld P.W., Riechel T.L., 1998. J. Agric. Food Chem., 46, 1887.
Harborne J.B., Williams C.A., 2000. Phytochemistry, 55, 481.

Harborne J.B., Boardley M., Frost S., Holm G., 1986. Plant Syst. Evol., 154, 251.
Hatcher D.W., Kruger J.E., 1997. Simple phenolic acids in flours prepared from Canadian wheat: Relationship to ash content, color, polyphenol oxidase activity. Cereal Chem., 74, 337–343.
Hernanz D., Nunez V., Sancho A.I., Faulds C.B., Williamson G., Bartolomé B., Gómez-Cordovés C., 2001. Hydroxycinnamic acids and ferulic acid dehydrodimers in barley and processed barley. Journal of Agriculture and Food Chemistry, 49, 4884–4888.
Himmelsbach D.S., Barton F.E., I.I., McClung A.M., Champagne E.T., 2001. Protein and amylose contents of milled rice by NIRFT/Raman spectroscopy. Cereal Chem., 78, 488–492.
Himmelsbach D., Akin D., 1998. Near-infrared Fourier-transform Raman spectrocopy of flax (Linum usitatissimum L.) stems. J. Agric. Food Chem., 46, 991–998.
Hosseinian F.S., Li W., Beta T., 2008. Food Chem., 109, 916.
Hvattum E., 2002. Rapid Commun. Mass Spectrom., 16, 655.
Izydorczyk K., Billiarderis C.G., 1995. Cereal arabinoxylans: Advances in structure and physicochemical properties. Carbohydr. Polym., 28, 33–48.
Jacobs D.R., Slavin J., Marquart L., 1995. Nutr. Cancer, 22, 221.
Jacobs D.R., Meyer K.A., Kushi L.H., Folsom A.R., 1998. Am. J. Clin. Nutr., 68, 248.
Joven J., Dinelli G., Fernandez-Gutierrez A., 2008. Electrophoresis, 29, 2852.
Kacurakova M., Wellner N., Ebringerova A., Hromadkova Z., Wilson R.H., Belton P.S., 1999. Characterization of xylan-type polysaccharides and associated cell-wall components by FT-IR and FT-Raman spectroscopies. Food Hydrocoll., 13, 35–41.
Kampa M., Alexaki V.I., Notas G., Nifl A.P., Nistikaki A., Hatzoglu A., Bakogeorgou E., Kouimtzoglu E., Blekas G., Boskou D., Gravanis A., Castanas E., 2004. Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells: potential mechanisms of action. Breast Cancer Research, 6, R63–R74.
Kasum C.M., Jacobs D.R., Nicodemus K., Folsom A.R., 2002. Int. J. Cancer, 99, 267.
Kikuzaki H., Hisamoto M., Hirose K., Akiyama K., Taniguchi H., 2002. Antioxidant properties of ferulic acid and its related compounds. Journal of Agriculture and Food Chemistry, 50, 2161–2168.
Kim K.H., Tsao R., Yang R., Cui S.W., 2006a. Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. Food Chemistry, 95, 466–473.
Kim K.-H., Tsao R., Yang R., Cui S.W., 2006b. Food Chem., 95, 466.
Kivilompolo M., V. Obůrka, Hyötyläinen T., 2008. Anal. Bioanal. Chem., 391, 373.
Lachman J., Capouchová I., 2006. Composition and contribution of major antioxidants of selected cereals to their nutrition quality. Scientia Agriculturae Bohemica, 37, 172–180.
Lachman J., Dudjak J., Orsák M., Pivec V., 2003. Plant Soil Environ., 49, 1.
Lam T.B.-T., Iiyamat K., Stone B.A., 1990. Phytochemistry, 29, 429.
Lee Y.T., Don M.J., Liao Ch.H., Chiou H.W., Chen Ch.F., Ho L.K., 2005. Effects of phenolic acid esters and amides on stimulus-induced reactive oxygen species production in human neutrophils. Clinica Chimica Acta, 352, 135–141.
Lempereur I., Rouau X., Abecassis J., 1997. J. Cereal Sci., 25, 103.
Lewis I.R., Edwards H.G.M., eds., 2001. Pages 35–36, [in:] Handbook of Raman Spectroscopy. Prac. Spectrosc., 28. Marcel Dekker: New York.
Li W., Shan F., Sun S., Corke H., Beta T., 2005. J. Agric. Food Chem., 53, 8533.
Liu L., Zubik L., Collins F.W., Marko M., Meydani M., 2004b. The antiatherogenic potential of oat phenolic compounds. Atherosclerosis, 175, 39–49.
Liu R.H., 2004a. J. Nutr., 134, 3479S.
Liyana-Pathirana C.-M., Shahidi F., 2005. J. Agric. Food Chem., 53, 2433.
Ma C.Y., Phillips D.L., 2002. Review: FT-Raman spectroscopy and its application in cereal science. Cereal Chem., 79, 171–177.
Martınez-Valverde I., Periago M.J., Provan G., Chesson A., 2002. J. Sci. Food Agric., 82, 323.

Mateos R., Goya L., Bravo L., 2006. Uptake and metabolism of hydroxycinnamic acids (chlorogenic, caffeic, and ferulic acids) by HepG2 cells as a model of the human liver. Journal of Agriculture and Food Chemistry, 54, 8724–8732.
Mattila P., Pihlava J.M., Hellstrom J., 2005. J. Agric. Food Chem., 53, 8290.
Mattila P., Pihlava J.M., Hellström J., 2005. Content of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. journal of Agriculture and Food Chemistry, 53, 8290–8295.
Matus-Cádiz M.A., Daskalchuk T.E., Verma B., Puttick D., Chibbar R.N., Gray G.R., Perron C.E., Tyler R.T., Hucl P., 2008. J. Agric. Food Chem., 56, 1644.
Mazza G., Gao L., [in:] E.-S.M. Abdel-Aal, P.J. Peter (Eds.), Specialty Grains for Food and Feed, AACC, St. Paul, MN, 2005, 313.
McCallum J.A., Walker J.R.L., 1989. Cereal Chem., 67, 282.
McCallum, J.A., Walker J.R.L., 1990. Proanthocyanidins in wheat bran. Cereal Chem., 67, 282–285.
Meyer K.A., Kushi L.H., Jacob D.R., Slavin J., Sellers T.A., Folsom A.R., 2000. Am. J. Clin. Nutr., 71, 921.
Miyamoto T., Everson E.H., 1958. Biochemical and physiological studies of wheat seed pigmentation. Agron. J., 50, 733–734.
Mpofu A., Sapirstein H.D., Beta T., 2006. J. Agric. Food Chem., 54, 1265.
Nicodemus K.K., Jacobs D.R., Folsom A.R., 2001. Cancer Causes Control, 12, 917.
O'Kennedy R., Thornes R.D. (Eds.), 1997. Coumarins—Biology, Applications and Mode of Action, John Wiley & Sons Ltd, Chichester.
Olenichenko N.A., Ossipov V.I., Zagoskina N.V., 2006. Russian J. Plant Physiol., 53, 495.
Onyeneho S.N., Hettiarachchy N.S., 1992. J. Agric. Food Chem., 40, 1496.
Orsák M., Lachman J., Vejdová M., Pivec V., Hamouz K., 2001. Changes of selected secondary metabolites in potatoes and buckwheat caused by UV, γ- and microwave irradiation. Rostlinná Výroba, 47, 493–500.
Pẽnalvo J.L., Haajanen K.M., Botting N., Adlercreutz H., 2005. J. Agric. Food Chem., 53, 9342.
Peterson D.M., 2001. Oat antioxidants. Journal of Cereal Science, 33, 115–129.
Peterson D.M., Hahn M.J., Emmons C.L., 2002. Oat avenanthramides exhibit antioxidant activities in vitro. Food Chemistry, 79, 473–478.
Petit-Benvegnen M.-D., Saulnier L., Rouau X., 1998. Solubilization of arabinoxylans from isolated water-unextractable pentosans and wheat flour doughs by cell-wall-degrading enzymes. Cereal Chem., 75, 551–556.
Peyron S., Surget A., Mabille F., Autran J.C., Rouau X., Abecassis J., 2002. J. Cereal Sci., 36, 199.
Peyron S., Abecassis J., Autran J.-C., Rouau X., 2001. Enzymatic oxidative treatments of wheat bran layers: Effects on ferulic acid composition and mechanical properties. J. Agric. Food Chem., 49, 4694–4699.
Phillips D.L., Liu H., Pan D., Corke H., 1999b. General application of Raman spectroscopy for the determination of level of acetylation in modified starches. Cereal Chem., 76, 439–443.
Phillips D.L., Xing J., Chong C.K., Corke H., 2001. Quantitative analysis of benzyl modification in waxy maize starch by Fourier transform Raman spectroscopy. Cereal Chem., 78, 629–631.
Phillips D.L., Xing J., Liu H., Pan D., Corke H., 1999a. Potential use of Raman spectroscopy for determination of amylose content in maize. Cereal Chem., 76, 821–823.
Piironen V., Lampi A.-M., Ekholm P., Salmenkallio-Marttila M., Liukkonen K.-H., [in:] Khan K., Shewry P.R. (Eds.), Wheat: Chemistry and Technology, 4th ed., 179–222.
Piot O., Autran J.-C., Manfait M., 2000. Spatial distribution of protein and phenolic constituents in wheat grain as probed by confocal microspectroscopy. J. Cereal Sci., 32, 57–71.
Piot O., Autran J.-C., Manfait M., 2002. Assessment of cereal quality by micro-Raman analysis of the grain molecular composition. Appl. Spectrosc., 56, 1132–1138.

Pussayanawin V., Wetzel D.L., Fulcher R.G., 1988. Fluorescence detection and measurement of ferulic acid in wheat milling fractions by microscopy and HPLC. J. Agric. Food Chem., 36, 515–520.
Ram M.S., Dowell F., Seitz L., Lookhart G., 2002. Development of standard procedures for a simple, rapid test to determine wheat color class. Cereal Chem., 79, 230–237.
Rao R.S.P., Muralikrishna G., 2004. Non-starch polysaccharides-phenolic acid complexes from native and germinated cereals and millet. Food Chemistry, 84, 527–531.
Rice-Evans C.A., Miller N.J., Paganga G., 1997. Trends Plant Sci., 2, 152.
Robards K.J., 2003. Chromatogr. A 1000, 657.
Robbins R.J., 2003. J. Agric. Food Chem., 51, 2866.
Run-Cang Sun, Xiao-Feng Sun, Shi-Hong Zhang, 2001. Quantitative determination of hydroxycinnamic acids in wheat, rice, rye, and barley straws, maize stems, oil palm frond fiber, and fast-growing poplar wood. Journal of Agriculture and Food Chemistry, 49, 5122–5129.
Rybka K., Sitarski J., Raczynska-Bojanowska K., 1993. Ferulic acid in rye and wheat grain and grain dietary fiber. Cereal Chem., 70, 55–59.
Sancho A.I., Bartolomé B., Gómez-Cordovés C., Williamson G., Faulds C.B., 2001. Release of ferulic acid from cereal residues by barley enzymatic extracts. Journal of Cereal Science, 34, 173–179.
Saulnier L., Sado P.-E., Branlard G., Charmet G., Guillon F., 2007. J. Cereal Sci., 46, 261.
Scalbert A., Monties B., Lallemand J.-Y., Guitet E., 1985. Ether linkage between phenolic acid and lignin fractions from wheat straw. Phytochemistry, 24, 1359–1362.
Seitz L.M., 1989. Stanol and sterol esters of ferulic and p-coumaric acids in wheat, corn, rye, and triticale. J. Agric. Food Chem., 37, 662–667.
Seitz L.M., 1992. Identification of 5-(2-oxoalkyl)resorcinols and 5-(2-oxoalkenyl) resorcinols in wheat and rye grains. J. Agric. Food Chem., 40, 1541–1546.
Seitz L.M., Love G.R., 1987. Identification and determination of alkylresorcinols and related compounds in cereal grains and milling fractions. (Abstr.) Cereal Foods World, 32, 651.
Servili M., Baldioli M., Selvaggini R., Miniati E., Macchioni A., Montedoro G., 1999. J. Am. Oil Chem. Soc., 76, 873.
Sidhu J.S., Kabir Y., Huffman F.G., 2007. Int. J. Food Prop., 10, 231.
Singleton V.L., Rossi J.A., 1965. Am. J. Enol. Vitic., 16, 144.
Sivakesava S., Irudayaraj J., Demirci A., 2001. Monitoring a bioprocess for ethanol production using FT-MIR and FT-Raman spectroscopy. J. Ind. Microbiol. Biotechnol., 26, 185–190.
Smigel K., 1992. J. Natl. Cancer Inst., 84, 80.
Soine T.O., J. 1964. Pharm. Sci., 53, 231.
Sun R., Lawther J.M., Banks W.B., 1996. J. Agric. Food Chem., 44, 3965.
Thompson L.U., 1994. Crit. Rev. Food Sci. Nutr., 34, 473.
Velioglu Y.S., Mazza G., Gao L., Oomah B.D., 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grains products. journal of Agriculture and Food Chemistry, 46, 4113–4117.
Waldron K.W., Parr A.J., Ng A., Ralph J., 1996. Phytochem. Anal., 7, 305.
Weidner S., Amarowicz R., Karamac M., Fraczek E., 2000. Changes in endogenous phenolic acids during development of Secale cereale caryopses and after dehydration treatment of unripe rye grains. Plant Physiology and Biochemistry, 38, 595–602.
Wood P.J., 2005, [in:] E.-S.M. Abdel-Aal, P.J. Peter (Eds.), Specialty Grains for Food and Feed, AACC, St. Paul, MN, 367.
Wu H., Haig T.J., Pratley D., Lemerle M., 1999. An. J. Chromatogr. A 864, 315.
Yao L.H., Jian Y.M., Shi J., Tomas-Barberan F.A., Datta N., Singanusong R., Chen S.S., 2004. Plant Foods Hum. Nutr., 59, 113.
Yeh C.T., Shih P.H., Yen G.C., 2004/ Synergistic effect of antioxidant phenolic acids on human phenolsulfotransferase activity. Journal of Agriculture and Food Chemistry, 52, 4139–4143.

Yeh C.T., Yen G.C., 2006. Modulation of hepatic phase II phenol sulfotransferase and antioxidant status by phenolic acids in rats. Journal of Nutritional Biochemistry, 17, 561–569.
Yu J., Vasanthan T., Temelli F., 2001. Analysis of phenolic acids in barley by high-performance liquid chromatography. Journal of Agriculture and Food Chemistry, 49, 4352–4358.
Yu L., Haley S., Perret J., Harris M., Wilson J., Qian M., 2000. J. Agric. Food Chem., 50, 1619.
Zhang W.-D., Qi L.-W., Yang X.-L., Huang W.-Z., Li P., Yang Z.-L., 2008. Rapid Commun. Mass Spectrom., 22, 2677.
Zhishen J., Mengcheng T., Jianming W., 1999. Food Chem., 64, 555.
Zielinski H., Kozlowska H., 2000. Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. journal of Agriculture and Food Chemistry, 48, 2008–2016.
Zielinski H., Kozlowska H., 2008. J. Agric. Food Chem., 48.

11

THE EFFECT OF CULTIVATION INTENSITY ON FATTY ACID COMPOSITION IN GRAIN, FLAKES AND BRAN OF WINTER WHEAT (*TRITICUM AESTIVUM* L.) – PRELIMINARY STUDY

Introduction

During the last decade, whole grain cereal products have captured scientific and public attention as a human nutrition basis and possible means to maintain and improve health condition. Epidemiological evidence implicates a low dietary intake of plant fiber as an etiological factor in diseases such as colon cancer, appendicitis, diverticular disease and ischemic heart disease [Hai 2007, He et al. 2010, Sapfer et al. 2000, Slavin 2003]. Diet rich in wheat bran can effect on glycemic control and risk factors for cardiovascular disease in type 2 diabetes, LDL oxidation and free radicals, as well as on the development of mammary tumors [Jenkins et al. 2002, Yu et al. 2005, Zile et al. 1998, Ou et al. 2007]. In accordance with the principles of the food pyramid developed by the National Food and Nutrition Institute low--processed cereal products are classified almost in the base section of the food pyramid and should be consumed at least in five portions per day (http://www.izz.waw.pl/index.php?option=com_content&view=article&id=7&Itemid=5&lang=pl#g).

The composition of wheat oil is beneficial for human health, because on polyunsaturated fatty acid (PUFA) high content. Advances in the knowledge concerning physiological functions of dietary PUFA have led to an increase of interest in the food sources of these nutrients. α-linolenic acid (ALNA) is a plant-derived n-3 fatty acid and is the precursor of the bioactive n-3 PUFAs. Together with linoleic acid (LA), the precursor of the bioactive n-6 PUFAs, is one of the two essential fatty acid in the human diet which cannot be synthesized by the human body. The are evidences form animal and human studies suggesting that ALNA can reduce blood pressure in hypertensives, inhibit lymphocyte proliferation, suppress the production of TNF and IL-1, and can act anti-thrombotic and anti-arrythmic [Burdge and Calder 2006, Pawlosky et al. 2001]. LA is the major dietary fatty acid regulating low-density lipoprotein (LDL)-cholesterol metabolism by downregulating LDL- cholesterol production and enhancing its clearance [Wijendran and Hayes 2004]. It is now recognized that dietary LA increases platelet response to aggregation, and suppresses the immune system [Renaud 1990, Simopoulos 1999]. Few studies provided indirect evidence for the preventive effect of wheat bran oil itself against intestinal tumorigenesis in animal models [Sang et al. 2006, Reddy et al. 2000].

Cereal products constitute one of the most important source of PUFA in human diet in Poland and Europe and state around 17% of daily intake of this nutrient [Stachura et al. 2009, Sioen et al. 2006].

The distribution of lipids within individual grains of cereal is uneven [Gąsiorowski 2004]. Most of lipids are concentrated in endosperm, germ and the aleurone cells of grain, which is commonly removed as bran during milling [Gąsiorowski 2004].

No research, however, has been published on how cultivation intensity affects the composition of fatty acid in wheat grain, flakes and bran.

Taking into account the current state of knowledge concerning the role of cereal products in prevention and dietary therapy of metabolic diseases, the authors decided to investigate whether cultivation intensity affects winter wheat grain (*Triticum aestivum* L.), flakes and bran fatty acid composition.

Material and methods

The initial material for study comprised grain of 3 new winter wheat cultivars Sukces, Kobiera and Rapsodia grown in 2 strain testing stations located in Lower Silesia – Zybiszów and Tomaszów Bolesławiecki in 2007. Investigated cultivars grew at two levels of cultivation intensity – lower (A1) and higher (A2). The higher level differed from the lower firstly in higher nitrogen dose by 40 kg, secondly in application of fungicide, growth regulator and foliage dressing (Tab. 1).

Table 1

Soil conditions of the investigation

Specification	Zybiszów	Tomaszów Bolesławiecki
Soil complex	2	3
Soil bonitation class	IIIa	IVa
Soil fertility in P2O5	25.8	21.8
Soil fertility in K2O	40.3	23.8
Soil fertility in Mg	8.8	9.2
Reaction in 1 M KCl	6.2	6.0
Nitrogen rates–A1(kg.ha-1)	114	129
Nitrogen rates–A2(kg.ha-1)	154	169
Herbicide	Granstar, Starane	Cougar
Fungicide A2	Juwell TT, Tango Star	Juwell TT, Caramba
Plant growth regulant –A2	Moddus	Moddus
Foliar fertilisation – A2	Basfoliar	Basfoliar

Obtaining of flakes

A two-stage hydrothermal processing was used to obtain wheat flakes. Samples of grain (300 g) were treated with water vapor evaporation (121^{o}C, 1 atm. in autoclave) and roasting (180^{o}C, 20 min. in laboratory dryer) to obtain wheat flakes. Samples were cooled at room temperature for 24 h and minced using laboratory hammer shredder after hydrothermal processing.

Obtaining of bran

Samples of grain (1000g) were humidified to 16% of moisture and left for 18 h, then were milled on a Tripette & Renaud & Chopin CD1 laboratory mill. Flour extraction rates averaged 60% in this study. The term "bran" in this study refers to all kernel components (fine and coarse bran), except the white flour fraction.

Lipid extraction

Extraction was performed using the Folch method [Folch et al. 1956].

Analysis of Fatty Acids

For grain, flakes and bran, the fatty acid composition of the total fat was examined using the gas chromatography method (Agilent 6890N). Methyl esters of the fatty acids (FAMEs) were obtained by means of esterification of fat samples according to the Prescha [Prescha et al. 2001] with modification. The chloroform extract of lipids was evaporated under nitrogen and fifty milligrams of lipids was saponified at 70°C for 1 h with 1 mL of 2 M KOH in 75% aqueous methanol. The unsaponifiable material was extracted two times with n-hexan and then removed. The potassium salts of the fatty acids were treated with 1 mL of 2 M HCl in water (at 70°C for 30 min). After the addition of saturated sodium chloride solution, the fatty acids were extracted with two 1 mL portions of hexane. The obtained fatty acids were esterified at 70°C using 1 mL of 0.5 M KOH for 30 min and then 1 mL of 1.25 M HCl for 30 min (both solutions in anhydrous methanol). The obtained FAMEs were extracted with two 1 mL portions of hexane as described previously. The methyl esters mixture was separated on a capillary column, HP 88 (100 m - 0.25 mm). Hydrogen was used as a carrier, and the separation was carried out at a temperature programmed from 165°C (for 10 min) to 240°C; the temperature increased at a rate of 2°C/min. The identification of particular fatty acids was accomplished by comparison with external standards. Fatty acid profile was recorded as a percentage of total fatty acid identified.

Statistical analysis

All data are presented as means with their standard deviation (mean ± SD). Statistical analyses were done using Statistica v 9.0 program. Data were compared using the

NIR test. A *P* value of less than 0.05 (P<0.05) was taken as the lowest level of statistical significance.

Results and discussion

The effect of tillage intensity on winter wheat grain (*Triticum aestivum* L.), flakes and bran fatty acid composition were investigated in this study.

The results of investigations of the changes in winter wheat grain, flakes and bran fatty acid composition are summarized in Tables 2–5.

Table 2

The sum of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in winter wheat grain, flakes and bran (% of all fatty acids, mean ± SD)

	Σ SFA				Σ MUFA				ΣPUFA			
	Zybiszów		Tomaszów Bol.		Zybiszów		Tomaszów Bol.		Zybiszów		Tomaszów Bol.	
Cultivar	A1	A2	A1	**A2**	**A1**	**A2**	**A1**	**A2**	**A1**	**A2**	**A1**	**A2**
GRAIN												
Sukces	19.59 ± 0.13	20.46 ± 0.55	18.96 ± 0.60	20.02 ± 0.21	14.35 ± 0.36	15.49 ± 0.10	14.89 ± 1.05	18.76 ± 0.78	62.81 ± 0.22	60.98 ± 0.72	63.43 ± 0.39	51.54 ± 0.44
Kobiera	20.10 ± 1.05	22.87 ± 3.82	18.60 ± 0.14	20.50 ± 0.48	19.34 ± 1.26	20.45 ± 1.51	17.15 ± 0.08	21.37 ± 0.20	57.50 ± 2.52	52.30 ± 0.36	61.64 ± 0.00	54.01 ± 0.36
Rapsodia	20.24 ± 0.27	19.82 ± 0.19	20.61 ± 0.92	20.77 ± 0.66	18.90 ± 2.25	16.61 ± 0.38	19.74 ± 1.08	19.02 ± 2.16	58.26 ± 2.04	60.34 ± 0.14	56.24 ± 0.01	56.98 ± 1.24
FLAKES												
Sukces	18.26 ± 0.92	18.04 ± 1.22	18.71 ± 0.23	20.24 ± 0.12	14.48 ± 1.38	13.98 ± 0.52	14.02 ± 0.25	14.58 ± 0.30	65.10 ± 2.60	65.88 ± 0.41	64.95 ± 0.66	62.59 ± 0.42
Kobiera	17.19 ± 0.10	17.61 ± 0.08	17.69 ± 0.27	18.27 ± 0.13	17.96 ± 0.64	16.72 ± 1.06	17.38 ± 0.17	17.24 ± 0.13	62.68 ± 0.88	63.33 ± 1.97	62.77 ± 0.38	61.17 ± 0.50
Rapsodia	17.46 ± 0.29	18.39 ± 0.17	17.82 ± 0.33	17.98 ± 0.53	16.32 ± 0.10	15.93 ± 0.40	16.39 ± 0.57	16.48 ± 0.38	63.95 ± 0.07	63.48 ± 0.20	63.03 ± 0.80	63.10 ± 0.06
BRAN												
Sukces	16.87 ± 0.12	18.76 ± 0.00	19.31 ± 0.29	19.63 ± 0.30	14.07 ± 0.53	13.54 ± 0.09	13.01 ± 0.60	14.43 ± 0.86	66.78 ± 1.02	65.16 ± 0.62	65.18 ± 0.74	63.49 ± 1.34
Kobiera	14.38 ± 0.43	19.21 ± 0.82	17.73 ± 0.27	18.23 ± 0.06	18.64 ± 0.41	17.38 ± 0.49	17.03 ± 0.16	16.19 ± 0.51	64.47 ± 0.04	60.96 ± 1.57	63.06 ± 0.02	63.43 ± 0.75
Rapsodia	19.24 ± 0.48	18.13 ± 0.01	17.34 ± 0.64	18.61 ± 0.29	17.93 ± 1.38	16.34 ± 0.69	16.81 ± 0.54	16.63 ± 1.01	60.37 ± 2.17	63.29 ± 0.86	62.77 ± 1.30	62.43 ± 1.58

A1 – lower level of cultivation intensity
A2 – higher level of cultivation intensity

The composition of initial material fatty acids was typical for wheat oil [Gąsiorowski 2004]. The average content of saturated fatty acids (SFA) was about 20,21%; 18,13% and 18,12% of all fatty acids in wheat grain, flakes and bran respectively (Tab. 2). Taking into

Table 3

The content of saturated fatty acids in winter wheat grain, flakes and bran calculated for cultivar, level of tillage intensity and place of cultivation (% of all fatty acids, mean ± SD)

Characteristic		C 16:0			C 18:0		
		GRAIN	FLAKES	**BRAN**	**GRAIN**	**FLAKES**	**BRAN**
Cultivar	Sukces	17.57 ± 0.70	**17.35 ± 0.84**	**17.02 ± 0.75**	2.18 ± 0.53	**1.45 ± 0.30**	**1.30 ± 0.19**
	Kobiera	17.98 ± 1.05	16.37 ± 0.36	16.10 ± 1.96	2.58 ± 1.29	1.32 ± 0.16	1.27 ± 0.15
	Rapsodia	18.15 ± 0.54	16.61 ± 0.46	17.40 ± 1.13	2.20 ± 0.23	1.30 ± 0.15	1.24 ± 0.16
p		n.s	**0.0031**	n.s	n.s	n.s	n.s
Level of tillage intensity	A1	17.76 ± 0.70	16.51 ± 0.52	16.51 ± 1.80	1.92 ± 0.35	1.33 ± 0.18	1.28 ± 0.18
	A2	18.04 ± 0.89	17.04 ± 0.79	17.17 ± 0.88	2.69 ± 0.94	1.38 ± 0.25	1.27 ± 0.15
p		n.s	n.s	n.s	n.s	n.s	n.s
Strain Testing Station	Zybiszów	18.20 ± 0.79	16.71 ± 0.58	17.19 ± 0.84	2.3 ± 0.75	1.19 ± 0.10	1.28 ± 0.14
	Tomaszów Bol.	17.60 ± 0.72	16.92 ± 0.78	16.49 ± 1.81	2.3 ± 0.60	1.53 ± 0.17	1.27 ± 0.19
p		n.s	n.s	n.s	n.s	**0.00009**	n.s
Mean		17.9	16.8	16.8	2.3	1.36	1.27

n.s – not statistically significant difference

account the composition of individual SFA the palmitic acid was present in the largest quantities: average 17,9% in grain and by 16,8% of all fatty acids in flakes and bran. The stearic acid was present in smaller quantities: average 2,3% in grain; 1,36% in flakes and 1,27% of all fatty acids in bran (Tab. 3). The content of palmitic acid showed in this study was slightly lower in comparison to results obtained by other authors [Konopka et al. 2006, Armanino et al. 2002, Ruibal-Mendieta et al. 2004]. According to Konopka [Konopka et al. 2006], the average content of palmitic acid in grain six cultivars of winter wheat ranged 18,9-20%, by Armanino [Armanino et al. 2002] the average content of this acid in cultivars from Italy, Germany and Great Britain was 18,66% and by Ruibal-Mendieta [Ruibal-Mendieta et al. 2004] was 19,1%. The content of palmitic acid obtained by Rotkiewicz [Rotkiewicz et al. 2004] in grain of three polish cultivars ranged 16,21–17,60% and was lower than those obtained in current study. However, stearic acid content of grain obtained in the current study was 3–4 times higher in comparison with those obtained by other authors [Konopka et al. 2006, Rotkiewicz et al. 2004, Ruibal-Mendieta et al. 2004]. Such big difference in results could by connected with different agrotechnical conditions and cultivar characteristics.

The composition of SFA in studied material was rather independent on cultivation conditions. Only in case of flakes, differences in amount of C 16:0 between cultivars and in amount of C 18:0 between strain testing stations were statistically significant.

Table 4

The content of monounsaturated fatty acids in winter wheat grain, flakes and bran calculated for cultivar, level of tillage intensity and place of cultivation (% of all fatty acids, mean ± SD)

Characteristic		C 16:1			C 18:1			C 20:1		
		GRAIN	FLAKES	BRAN	**GRAIN**	**FLAKES**	**BRAN**	**GRAIN**	**FLAKES**	**BRAN**
Cultivar	Sukces	0.42 ± 0.17	0.25 ± 0.06	0.26 ± 0.07	14.89 ± 1.71	13.51 ± 0.64	16.07 ± 0.91	0.55 ± 0.05	0.49 ± 0.02	0.58 ± 0.02
	Kobiera	0.46 ± 0.16	0.24 ± 0.05	0.24 ± 0.03	18.44 ± 1.67	16.44 ± 0.65	16.38 ± 0.94	0.65 ± 0.04	0.63 ± 0.01	0.68 ± 0.04
	Rapsodia	0.41 ± 0.08	0.21 ± 0.03	0.23 ± 0.05	17.63 ± 1.71	15.56 ± 0.35	12.99 ± 0.70	0.51 ± 0.03	0.49 ± 0.01	0.52 ± 0.02
p		n.s	n.s	n.s	**0.00042**	**0.000000**	**0.000000**	**0.000001**	**0.000000**	**0.000000**
Level of tillage intensity	A1	0.37 ± 0.09	0.23 ± 0.03	0.24 ± 0.06	16.39 ± 2.40	15.32 ± 1.53	15.30 ± 2.19	0.57 ± 0.07	0.54 ± 0.07	0.61 ± 0.08
	A2	0.50 ± 0.15	0.25 ± 0.06	0.24 ± 0.05	17.53 ± 2.12	15.02 ± 1.23	15.00 ± 1.29	0.57 ± 0.07	0.54 ± 0.07	0.58 ± 0.06
p		**0.017**	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
Strain Testing Station	Zybiszów	0.40 ± 0.10	0.21 ± 0.03	0.24 ± 0.04	16.56 ± 2.34	15.15 ± 1.47	14.01 ± 2.93	0.55 ± 0.06	0.53 ± 0.07	0.59 ± 0.06
	Tomaszów Bol.	0.47 ± 0.17	0.26 ± 0.05	0.25 ± 0.06	17.42 ± 2.16	15.20 ± 1.32	15.45 ± 1.96	0.60 ± 0.07	0.54 ± 0.07	0.60 ± 0.07
p		n.s	**0.010**	n.s	n.s	n.s	n.s	n.s	n.s	n.s
Mean		0.43	0.23	0.24	16.99	15.17	14.73	0.57	0.53	0.59

n.s – not statistically significant difference

Table 5

The content of polyunsaturated fatty acids in winter wheat grain, flakes and bran calculated for cultivar, level of tillage intensity and place of cultivation (% of all fatty acids, mean ± SD)

Characteristic		C 18:2			C 18:3		
		GRAIN	**FLAKES**	**BRAN**	**GRAIN**	**FLAKES**	**BRAN**
Cultivar	Sukces	55.43 ± 4.75	60.10 ± 1.61	57.54 ± 1.55	4.25 ± 0.40	4.52 ± 0.12	4.67 ± 0.25
	Kobiera	52.52 ± 3.55	58.19 ± 1.24	58.61 ± 1.55	3.84 ± 0.45	4.29 ± 0.16	4.36 ± 0.38
	Rapsodia	54.00 ± 1.94	58.93 ± 0.58	60.30 ± 1.40	3.94 ± 0.22	4.45 ± 0.15	4.84 ± 0.21
p		n.s	**0.005**	**0.001**	**0.039**	**0.006**	**0.003**
Level of tillage intensity	A1	55.8 ± 2.71	59.18 ± 1.29	58.84 ± 1.86	4.17 ± 0.40	4.48 ± 0.16	4.65 ± 0.41
	A2	52.17 ± 3.64	58.89 ± 1.58	58.76 ± 1.92	3.85 ± 0.34	4.36 ± 0.16	4.60 ± 0.27
p		**0.011**	n.s	n.s	**0.046**	n.s	n.s
Strain Testing Station	Zybiszów	54.82 ± 3.25	59.74 ± 1.40	58.55 ± 1.12	3.87 ± 0.40	4.32 ± 0.17	4.83 ± 0.18
	Tomaszów Bol.	53.15 ± 3.97	58.41 ± 1.13	59.06 ± 2.39	4.15 ± 0.35	4.52 ± 0.10	4.41 ± 0.35
p		n.s	**0.018**	n.s	n.s	**0.003**	**0.001**
Mean		53.98	59.07	58.80	4.01	4.42	4.62

n.s – not statistically significant difference

The monounsaturated fatty acid (MUFA) was the next appointed group of lipids, which range from 15,95% of flakes to 18% of all fatty acids of grain (Tab. 2). The monounsaturated fatty acid with the highest content in initial material, was oleic acid C 18:1 – mean 95% of MUFA. The mean content of the oleic acid of grain was 16,99%, flakes – 15,17% and in bran – 14,73% of all fatty acids. The palmito-oleic and eikosaenoic acid were present in decisively smaller quantities: average 0,43% and 0,57% in grain; 0,23% and 0,53% in flakes and 0,24% and 0,59% of all fatty acids in bran, respectively (Tab. 4).

The oleic and eikosaenoic acids contents of grain obtained in the current study were comparable with those obtained by other authors [Rotkiewicz et al. 2004, Armanino et al. 2002]. However, oleic acid content was higher than those obtained by Konopka – 11,2–11,8 % of oleic acid of grain six cultivars of winter wheat and Ruibal-Mendieta et al. – 10,9% (Konopka et al., 2006, Ruibal-Mendieta et al., 2004). Likewise in the palmito-oleic acid case, content in grain obtained in the current study was higher than those obtained by other authors [Konopka et al. 2006, Armanino et al. 2002]. The content of this acid obtained by Konopka

[Konopka et al. 2006] in grain six cultivars of winter wheat was in range 0,01–0,05%, and mean content obtained by Armanino [Armanino et al. 2002] was 0,16%.

The composition of MUFA in studied material was rather independent on cultivation conditions. Only in case of grain, content of palmito-oleic acid enlarged from 0,37% on A1 tillage intensity to 0,50% of all fatty acids on higher tillage intensity, being statistically important. The range of significant changes in oleic and eikosaenoic acids contents of grain, flakes and bran was depended on cultivar characteristics.

Analyzing the quantity of polyunsaturated fatty acid in study design it was affirmed, that PUFA constitute 53,25; 63,50 and 63,19% of all fatty acids of wheat grain, flakes and bran respectively (Tab. 2).

Taking into account the composition of individual PUFA the linoleic acid was present in the largest quantities: average 53,98% of grain; 59,07% of flakes and 58,80% of all fatty acids of bran. The linolenic acid was present in smaller quantities: average 4,01% of grain; 4,42% of flakes and 4,62% of all fatty acids of bran (Tab. 5).

The linolenic fatty acid content of grain obtained in the current study was comparable with those obtained by other authors [Rotkiewicz et al. 2004, Konopka et al. 2006, Ruibal-Mendieta et al. 2004]. However, linoleic fatty acid content was slightly lower, around 3– 9%, than those obtained by other authors [Rotkiewicz et al. 2004, Konopka et al. 2006, Ruibal-Mendieta et al. 2004].

The polyunsaturated fatty acid contents in studied material were affected by cultivars, tillage intensity and cultivation place. The effect of cultivar characteristics on both linoleic and linolenic fatty acids contents was significant in all studied material, except linoleic fatty acid of grain. The decrease of both linoleic and linolenic fatty acids contents in grain was significant and depended on tillage intensity, on the other hand changes in content of these fatty acids of flakes and bran were significantly affected by cultivation place, except linoleic fatty acid of bran.

According to introduced results of present investigations, changes in composition of fatty acids of grain, flakes and bran were observed. The decrease of saturated and monounsaturated fatty acids content simultaneously with polyunsaturated fatty acid content increase of flakes compared with grain were observed. This changes could induce flakes hydrothermal producing process. Under the influence of water and temperature followed amylose-lipid and protein-lipid complexes formation [Bhatnagar et al.1994, Kołakowska and Sikorski 2011, Kaur and Singh 2000]. During hydrolysis in FAMEs obtaining process, protein-lipid complexes should be dissolve and probably only a part of amylose-lipid complexes is decompose. Most of the amylose-lipid complexes are create from saturated and monounsaturated fatty acids [Bhatnagar et al. 1994, Kaur and Singh 2000]. It could be explanation of changes in composition of fatty acids of grain and flakes. Similar changes in composition of fatty acids of grain and bran were observed. However, it was caused rather by specific fatty acids distribution in grain than by chemical reactions.

The cultivar characteristics had the strongest effect on composition of fatty acids in analyzed material, especially in PUFAs composition. Differences in the fatty acids compositions is strongly correlated with wheat cold-sensitive genotype. Many studies shows that the quantity of unsaturated fatty acids in the membranes increases as the temperature declines and the degree of unsaturation of the membrane fatty acids is correlated with frost tolerance [Szalai et al. 2000, Harwood 1996]. Cultivars analyzed in presented study characterized different frost tolerance degree, and this fact reflects in obtained results of fatty acid composition.

There is little knowledge on the genetics of fatty acid in wheat. For economic and nutritional reasons scientists try to construct quantitative trait locus (QTL) maps [Wang et al. 2011]. In this study, 40 QTLs for palmitic, stearic, oleic, linoleic, and linolenic acid content in wheat grain were detected on 16 chromosomes. The markers around these QTLs might be useful in wheat breeding programs aimed at germ oil [Wang et al. 2011].

Not only frost as an environmental stress factor can effect on fatty acid synthesis, but also the use of herbicides which inhibit different enzymes involved in this process. Components of herbicides effects on specific desaturases or elongases in fatty acid synthesis [Harwood 1996].

The use of fungicides, plant growth regulants, foliar fertilization in A2 tillage intensity in presented study, probably could also affect the fatty acids synthesis. However, as far as we know, the study on this aim have not been undertaken.

Conclusions

From the above results, it can be concluded that cultivation intensity effect on palmito--oleic fatty acid and PUFAs content in winter wheat grain only. The monounsaturated and polyunsaturated fatty acids content of grain, flakes and bran is mainly affected by cultivar characteristics. The saturated fatty acids content of wheat grain, flakes and bran is rather stable, regardless of varieties, tillage intensity and cultivation place.

References

Armanino C., de Acutis R., Festa M.R., 2002. Wheat lipids to discriminate species, varietes, geographical origins and crop years. Anal. Chim. Acta., 454, 315–326.

Bhatnagar S., Hanna M.A., 1994. Amylose-lipid complex formation during single-screw extrusion of various corn starches. Cereal Chem., 71(6), 582–587.

Burdge G.C., Calder P.C., 2006. Dietary a-linolenic acid and health-related outcomes: a metabolic perspective. Nutr. Res. Rev., 19, 26–52.

Folch J., Lees M., Sloan-Stanley G.H., 1956. A simple method for isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226, 497–509.

Gąsiorowski H., 2004. Wheat – chemistry and technology. PWRiL, Poznań, 188–204 (in Polish).

Hai L.R., 2007. Whole grain phytochemicals and health. J. Cereal Sci., 46, 207–219.

Harwood J.L., 1996. Recent advances in the biosynthesis of plant fatty acids. Biochem. Biophys. Acta 1301, 7–56.

He M., van Dan R.M., Rimm E., Hu F.B., Qi L., 2010. Whole-grain, cereal fiber, bran, and germ intake and the risk of all-cause and cardiovascular disease – specific morality among women with type 2 diabetes mellitus. Circulation, 121, 2162–2168.

http://www.izz.waw.pl/index.php?option=com_content&view=article&id=7&Itemid=5&lang=pl#g (accessed 29 Mai 2011)

Jenkins D., Kendall C., Augustin L., Martini M., Axelsen M., Faulkner D., Vidgen E., Parker T., Lau H., Connelly P., Teitel J., Singer W., Vandenbroucke A., Leiter L., Josse R., 2002. Effect of wheat bran on glycemic control and risk factors for cardiovascular disease in type 2 diabetes. Diabetes Care, 25, 1522–1528.

Kaur K., Singh N., 2000. Amylose-lipid complex formation during cooking of rice flour. Food Chem., 71(4), 511–517.
Kołakowska A., Sikorski Z.E., 2011. Chemical and functional properties of food lipids. CRC Press, New York, 458–473.
Konopka I., Czaplicki S., Rotkiewicz D., 2006. Differences in content and composition of free lipids and carotenoids in flour of spring and winter wheat cultivated in Poland. Food Chem., 95, 290–300.
Ou S., Jackson M., Jiao X., Chen J., Wu J., Huang X., 2007. Protection against oxidative stress in diabetic rats by wheat bran feruloyl oligosaccharides. J. Agric Food Chem., 55, 3191–3195.
Pawlosky R.J, Hibbeln J.R., Novotny J.A., Salem N., 2001. Physiological compartmental analysis of a-linolenic acid metabolism in adult humans. J. Lipid Res., 42, 1257–1265.
Prescha A., Siwędrych A., Biernat J., Szopa J., 2001. Increase in lipid content in potato tubers modified by 14–3–3 gene overexpression. J. Agric. Food Chem., 49, 3638–3643.
Reddy B.S., Hirose Y., Cohen L.A., Simi B., Cooma I., Rao C.V., 2000. Preventive potential of wheat bran fractions against experimental colon carcinogenesis: Implication for human colon cancer prevention. Cancer Res., 60, 4792–4797.
Renaud S., 1990. Linoleic acid, platelet aggregation and myocardial infarction. Atherosclerosis, 80, 255–256.
Rotkiewicz D., Konopka I., 2004. Charakterystyka lipidów ziarna pszenicy oraz wpływ ich przemian na jakość i wartość wypiekową mąki. Przegl. Zboż. Młyn., 8, 26–28.
Ruibal-Mendieta N.L., Dekeyser A., Delacriox D.L., Mignolet E., Larondelle Y., Meurens M., 2004. The oleate/palmitate ratio allows the distinction between wholemeals of spelt (*Triticum spelta* L.) and winter wheat (*T. aestivum* L.). J. Cereal Sci., 39, 413–415.
Sang S., Ju J., Lambert J.D., Lin Y., Hong J., Bose M., Wang S., Bai N., He K., Reddy B.S., Ho C-T., Li F., Yang C.S., 2006. Wheat bran oil and its fractions inhibit human colon cancer cell growth and intestinal tumorigenesis in $Apc^{min/+}$ mice. J. Agric. Food Chem. 54, 9792–9797.
Sapfer M.J., Hu F.B., Manson J.E., Rimm E.B., Willett W.C., 2000. Primary prevention of coronary heart disease in women through diet and lifestyle. N. Eng. J. Med., 343, 16–22.
Simopoulos A., 1999. Essential fatty acids in health and chronic disease. Am. J. Clin. Nutr., 70(suppl), 560S–9S;
Sioen I.A., Pynarrt I., Matthys C., de Backer G., van Camp J., de Henauw S., 2006. Dietary intake and food sources of fatty acids for Belgian women, focused on n-6 and n-3 polyunsaturated fatty acids. Lipids, 41(5), 415–422.
Slavin J., 2003. Why whole grains are protective: biological mechanisms. Proc. Nutr. Soc., 62, 129–134.
Stachura A., Pisulewski P.M., Kopeć A, Leszczyńska T., Bieżanowska-Kopeć R., 2009. Assessing the dietary intake level of total fats and fatty acids by high school students living in rural areas in the Beskid Żywiecki region. Żywność. Nauka. Technologia. Jakość, 5 (66), 119–131 (in Polish).
Szalai G., Janda T., Páldi E., Dubach J-P., 2001. Changes in the fatty acid unsaturation after hardening in wheat chromosome substitution lines with different cold tolerance. J. Plant Physiol., 158, 663–666.
Wanga Y-Y., Sunc X-Y., Zhaoa Y., Konga F-M., Guoa Y., Hanga G-Z., Pua Y-Y., Wuc K., Li S-S., 2011. Enrichment of a common wheat genetic map and QTL mapping for fatty acid content in grain. Plant Sci., 181, 65–75.
Wijendran V., Hayes K.C., 2004. Dietary n-6 and n-3 fatty acid balance and cardiovascular health. Ann. Rev. Nutr., 24, 597–615.
Yu L., Zhou K., Parry J., 2005. Inhibitory effects of wheat bran extracts on human LDL oxidation and free radicals. LWT Food Sci. Technol.,38, 463–470.
Zile M., Welsch C., Welsch M., 1998. Effect of wheat bran fiber on the development of mammary tumors in female intact and ovariectomized rats treated with 7,12-dimethylbenz(a)anthracene and in mice with spontaneously developing mammary tumors. Int. J. Cancer., 75, 439–443.

12

AN ATTEMPT TO USE COMPUTER VISION SYSTEMS (CVS) TO DETECT DFD DEFECT IN BEEF

Introduction

Basic criteria for evaluation of beef quality are its pH and color. Deviations from typical pH changes after a slaughter result in meat quality defects. Currently in Poland, meat taken from about 30% of young bull's carcasses show characteristics of meat with DFD. This defect is most common in such parts of carcasses as: the longest muscle and internal part of top round [Pospiech 1997, Wajda and Daszkiewicz 2001, Denaburski and Bąk 2002]. In comparison with normal meat, meat with DFD defect is characterized by higher pH, darker color and dense consistency. Its limited applicability for production of culinary meat is a result of less stable microbiological content arising from both high pH and dark unnatural color. For customer who buys beef, unnatural, dark red color is a sign of old meat. This is why a typical bright red color of beef indicates its freshness and that it is taken from young animals [Prokopiuk 2006]. It is a reason why the color of meat is a feature which should be evaluated quickly and objectively. Increasingly, computer vision systems are used for such objective evaluation of beef quality. Results achieved from using such systems enable to determine both meat color and its selected technological features, marbling and fat content in meat.

The aim of this study was an attempt to use computer vision systems (CVS) to detect DFD defect in beef obtained from young bull's carcasses.

Material and methods

Material for the study was samples obtained from top round from 40 young bull's carcasses. Meat samples were obtained from carcasses classified as meatiness class "O". Based on pH measurement [PN-ISO 2917 2001], 48 hours *post mortem,* meat was divided into two groups: normal (pH<5,8) and DFD meat (pH≥5,8). Next, color of meat was determined with the use of colorimetric method (in CIELab system) and computer vision systems (in three color models: RGB, HSV and HSL). Color measurement was made after 20 minutes of meat conditioning (in temp. of 8ºC) with the use of Minolta CM 2600 colorimeter (light source D_{65}, observer 2º, measurement head hole 8 mm) calibrated according

to whiteness standard (L* 99,18, a* -0,07, b* -0,05). In order to determine meat color with the use of computer vision systems, each sample was placed on measuring station and photographed. Standard conditions of taking photographs were assured by: incandescent lighting (4 matt bulbs 25 W each, color temperature of 2700K and color rendering index of light source (CRI) 90-100) and distance between lens and photographed meat surface (500 mm). Photos were taken with digital camera CANON EOS 350D in black matt measuring station with the use of light diffusion film. Digital image was processed and subjected to mathematical and statistical analysis in IMAGE ANALYZER software. The photos showed information of particular pixels of meat samples image – their color (R, G, B values from RGB model and V and L from HSV and HSL models; IMAGE ANALYZER guide, 2008).

The amount of drip loss was determined in 100 g of slices of meet stored in temp. of 4ºC for 24 h. The amount of thermal drip was determined in ground meat [Tyburcy 2006]. Achieved results were subjected to statistical analysis [Gawęcki and Wagner 1984] with the use of Statgraphics 4.1 software for level of significance $p \leq 0,05$.

Results and discussion

Average pH of normal meat was at the level of 5.6, whereas in case of meat with DFD – 5.9. Meat classification was made according to pH defined in literature. As Silva et al. [1999] and Viljoen et al. [2002] indicate, 5.8 is a limit value for normal meat and DFD meat. It was indicated in this analysis that meat content with DFD defect at the level of about 20% of the hole population. The lowest pH determined in beef was at the level of 5.5 what indicates that PSE defect was not in progress. As Miciński et al. [2005] indicate, significant causes of DFD defect in meat from young bulls are e.g.: ebullience and pre-slaughter stress. Wichłacz et al. [1995] and Lowe et al. [2004] state that there is a dependency between pH and color of meat. In case of meat with DFD defect there is a slight decrease of pH as a result of meat running out of glycogen too early what also triggers darker color of meat. Whereas in case of normal meat where glycolysis process is typical there is a decrease of pH to a level of 5,4–5,8 what, as a result, ensures typical red color of beef. Such dependencies were also found in these analysis where normal meat was characterized by significantly higher level of lightness. L* color value determined with the use of colorimetric method was at the level of 34.2 and was significantly higher by 3.6 in comparison with DFD meat in which the value was 30.6 (Tab. 1). However there were no significant differences between values of a* and b* values for both groups of beef. Similar dependencies were discovered during analysis conducted by Gašperlin et al [2000]. Absolute difference (between color of normal meat and DFD meat) was evaluated with the use of color values in CIELab system (Tab. 1). The following equation was applied:

$$\Delta E = \sqrt{(L^* - L)^2 + (a^* - a)^2 + (b^* - b)^2},$$

where

ΔE – absolute color difference,

L*, a*, b* – color values determined for normal meat; L, a, b – color values determined for meat with DFD defect.

Table 1

Chosen technological quality characteristics of analyzed meat and color values determined in CELab system and with the use of CVS method

	Normal meat		Meat with DFD	
	x	±s	x	±s
Drip loss [%]	0.6[a]	0.2	0.2[b]	0.09
Thermal drip [%]	9.2[a]	3.3	5.9[b]	3.6
L*	34.2[a]	2.5	30.6[b]	1.7
a*	13.2[a]	2.1	12.4[a]	1.1
b*	17.9[a]	2.2	17.9[a]	1.7
R	127.5[a]	10.4	119.3[a]	9.1
G	72.8[a]	8.1	68.9[a]	7.9
B	74.2[a]	8.6	69.0[a]	7.4
V	51.0[a]	3.4	44.2[b]	2.7
L	40.0[a]	3.0	34.3[b]	2.2

x ± s – average value ± standard deviation

a, b – averages in rows marked with the same letters are not significantly different at $p<0,05$

Criteria accepted by the International Commission on Illumination indicate that absolute color differences ΔE which are between 0 to 2 are indistinguishable and these between 2 and 3.5 are distinguishable to inexperienced observer, whereas in case of values over 3.5 there is a clearly visible difference between colors [Anonymous 1999]. The absolute color difference evaluated in these analysis was ΔE=3,7. It shows that normal meat was characterized by different color in comparison with the color of meat with DFD defect. However it is worth to indicate that the color of two analyzed quality groups differed mainly in its lightness (Tab. 1).

Beef samples obtained from young bull's carcasses with DFD defect symptoms were characterized by significantly lower amount of drip loss and thermal drip (Tab. 1) what corresponds to data included in literature.

Color values determined with the use of CVS method were used to classify beef into normal and DFD meat. In case of RGB model, color values determined with the use of CVS method were not significantly diversified in view of the type of analyzed meat. Although the R, G, B values determined in normal and DFD meat samples were similar, there is a clear tendency of growing values in case of normal meat samples (accordingly 127,5; 72,8 and 74,2 for normal meat and 119,3, 68,9 and 69,0 for meat with DFD; Tab. 1). Also the analysis of values directly characterizing lightness (V and L) showed that normal meat samples were characterized by significantly higher of V and L values (accordingly 51,0 and 40,0) in comparison with DFD meat (V:44,2 and L: 34,3, Tab. 1).

Results of many analysis indicate dependencies between pH and L* color lightness of meat. This work includes analysis of correlation between pH of analyzed meat and color values determined with the use of CVS method (V and L). Statistical analysis showed significant dependencies between meat pH and V and L color values determined with the use of CVS method. Calculated correlation and determination coefficients were at the level of r=-0,72* and R^2=52,0 for V and r=-0,70* i R^2=49,5 for L color value (Fig. 1 and 2).

It indicates a possibility o using color values determined with the use of CVS method for both estimating pH and distinguishing between quality groups of beef obtained from young bulls.

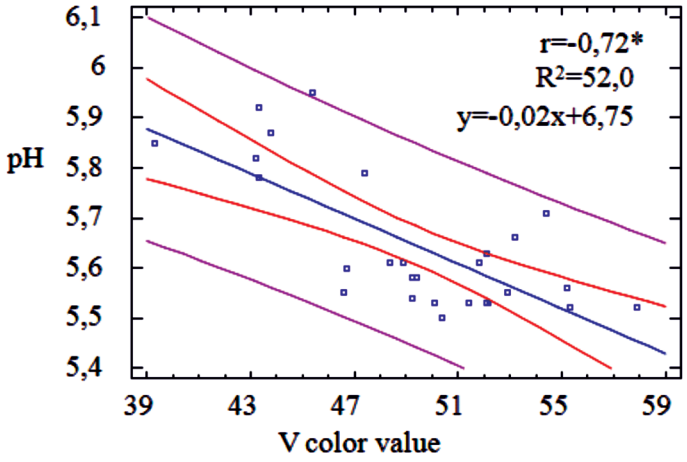


Fig. 1. Correlation between pH and V color value determined with the use of CVS method of analyzed beef

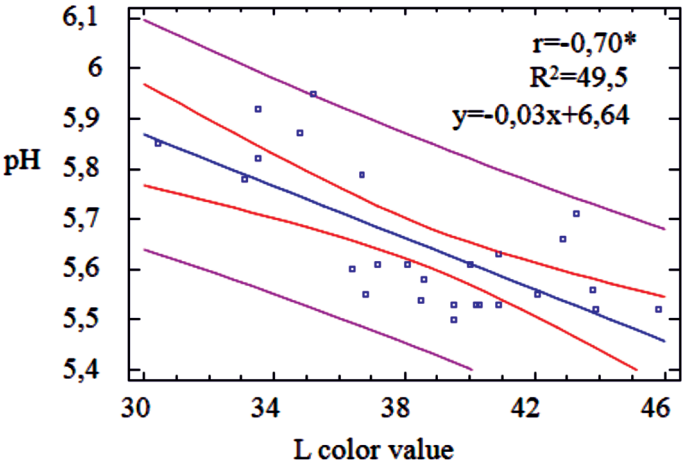


Fig. 2. Correlation between pH and V color value determined with the use of CVS method of analyzed beef

Conclusions

- Beef samples from young bull's carcasses with DFD defect were characterized with lower amount of drip and thermal loss. It was also found out that meat with DFD defect was characterized by darker color in comparison with normal meat.

- Irrespectively of the type of analyzed meat, R, G, B color values determined with the use of CVS method were not significantly diversified. Only the analysis of color values directly characterizing lightness (V and L) showed that normal meat samples were characterized with significantly higher values of these values. It indicates a possibility of using these values to classify meat into normal and meat with DFD defect.

Acknowledgements

This work is financially supported from the budget for science in 2010-2011, project Iuventus Plus No. 2010 004970

References

Anonim, 1999. Barwa i jakość. Heidelberg Druckmaschinen AG, Kurfursten-Anlage, 52–60.

Denaburski J., Bąk T., 2002. Najważniejsze przyczyny i sposoby zapobiegania występowaniu mięsa typu DFD. Gosp. Mięs., 5 (54), 28–31.

Gašperlin L., Žlender B., Abram V., 2000. Colour of normal and high pH beef heated to different temperatures as related to oxygenation. Meat Sci., 54, 391–398.

Gawęcki J., Wagner W., 1984. Podstawy metodologii badań doświadczalnych w nauce o żywieniu i żywności. Wydawnictwo Naukowe PWN, Warszawa.

Instrukcja programu Image Analyzer. 2008.

Lowe T.E., Devine C.E., Wells R.W., Lynch L.L., 2004. The relationship between post-mortem urinary catecholamines, meat ultimate pH, and shear force in bulls and cows. Meat Sci., 67, 251–260.

Miciński J., Klupczyński J., Ostoja H., Cierach M., Dymicka M., Łozicki A., Daszkiewicz T., 2005. Wpływ rasy i żywienia buhajków na wyniki klasyfikacji tusz w systemie EUROP oraz na ocenę tekstury mięsa. Żywność, Nauka, Technologia, Jakość, 3 (44), 147–156. PN-ISO 2917. 2001. Mięso i przetwory mięsne. Pomiar pH.

Pospiech E., 1997. Analiza przetwarzania mięsa o obniżonej jakości. Gosp. Mięs., 6 (49), 34–37.

Prokopiuk G., 2006. Produkcja dobrej jakości mięsa wołowego. Mięso i Wędliny, 8, 8–12.

Silva J.A., Patarata L., Martins C., 1999. Influence of ultimate pH on bovine meat tenderness during ageing. Meat Sci., 52, 453–459.

Tyburcy A., 2006. Charakterystyka właściwości technologicznych mięsa, [w:] Mitek M., Słowiński M. (red.). Wybrane zagadnienia z technologii żywności. Wydawnictwo SGGW, Warszawa, 271–285.

Viljoen H.F., de Kock H.L., Webb E.C., 2002. Consumer acceptability of dark, firm and dry (DFD) and normal pH beef steaks. Meat Sci., 61, 181–185.

Wajda S., Daszkiewicz T., 2001. Kulinarne mięso wołowe i ocena jego właściwości organoleptycznych. Gosp. Mięs., 9 (53), 18–22.

Wichłacz H., 1995. Wpływ postępowania z bydłem przed ubojem na przydatność mięsa do celów kulinarnych. Gosp. Mięs. 12 (47), 58–60.

AUTHORS

Biernat Jadwiga – Silesian Piasts University of Medicine in Wrocław, Poland
Bodkowski Robert – Wrocław University of Environmental and Life Sciences, Poland
Bulla Jozef – Nitra Slovak University of Agriculture, Slovakia
Buszewski Bogusław – Toruń Nicolaus Copernicus University, Poland
Chmiel Marta – Warsaw University of Life Sciences-SGGW, Poland
Dasiewicz Krzysztof – Warsaw University of Life Sciences-SGGW, Poland
Dobrzański Zbigniew – Wrocław University of Environmental and Life Sciences, Poland
Filipiak Marian – Poznań University of Economics, Poland
Florowski Tomasz – Warsaw University of Life Sciences-SGGW, Poland
Goldsztejn Magdalena – Puławy National Veterinary Research Institute, Poland
Golian Jozef – Nitra Slovak University of Agriculture, Slovakia
Grajewa Halina – Silesian Piasts University of Medicine in Wrocław, Poland
Grenda Tomasz – Puławy National Veterinary Research Institute, Poland
Gwiazdowska Daniela – Poznań University of Economics, Poland
Jamroz Dorota – Wrocław University of Environmental and Life Sciences, Poland,
Jasiczak Jan – Poznań University of Economics, Poland
Kikut-Ligaj Dariusz – Poznań University of Economics, Poland
Kowalik Jarosław – Olsztyn University of Warmia and Mazury, Poland.
Kukier Elżbieta – Puławy National Veterinary Research Institute, Poland
Kwiatek Krzysztof – Puławy National Veterinary Research Institute, Poland
Ligaj Marta – Poznań University of Economics, Poland
Ligor Magdalena – Toruń Nicolaus Copernicus University, Poland
Lochyński Stanisław – Wrocław University of Technology, Poland, Wrocław College of Physiotherapy, Poland
Łobacz Adriana – Olsztyn University of Warmia and Mazury, Poland
Marek Szołtysik – Wrocław University of Environmental and Life Sciences, Poland
Maršálková Lenka – Nitra Slovak University of Agriculture, Slovakia
Maruška Audrius – Kaunas Vytautas Magnus University, Lithuania
Nawracała Jerzy – Poznań University of Life Sciences, Poland
Nowakowski Piotr – Wrocław University of Environmental and Life Sciences, Poland
Nuc Katarzyna – Poznań University of Life Sciences, Poland
Patkowska-Sokoła Bożena – Wrocław University of Environmental and Life Sciences, Poland
Perkowski Juliusz – Poznań University of Life Sciences, Poland
Pękala Jolanta – Wrocław University of Technology, Poland
Pieczyńska Joanna – Silesian Piasts University of Medicine in Wrocław, Poland
Skolik Agnieszka – Poznań University of Economics, Poland
Słowiński Mirosław – Warsaw University of Life Sciences-SGGW, Poland
Steininger Mieczysław – Wrocław University of Technology, Wrocław,
Stuper Kinga – Poznań University of Life Sciences, Poland